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THE UNIVERSITY OF ALBERTA

AN EXPERIMENTAL STUDY OF ISOLATION PERFUSION AND INFUSION TECHNIQUES IN REGIONAL CANCER CHEMOTHERAPY

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

FACULTY OF MEDICINE
DEPARTMENT OF SURGERY
by
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ABSTRACT.

The technique of isolation perfusion chemotherapy of regionally confined neoplasms, presently used as an adjunct to surgery or as a means of palliation in far advanced cancer, has been attended with a varying tumor response in humans. Use of this technique and other methods of regional chemotherapy, e.g. intra-arterial infusion, has been largely uncontrolled and the results difficult to evaluate. Most related experimental work has been performed on the dog, but the lack of naturally occurring or transplantable tumors in this animal is inconvenient. An experimental technique was therefore devised by which the many variables associated with perfusion could be studied in terms of tumor response.

The technique provided a method whereby the circulation of the right hind limb of rats was isolated from the general body circulation. The isolated limb was perfused with donor rat blood at pressures below systemic pressure, so that the leak of perfused blood into the systemic circulation was negligible. Controlled alteration of the biochemical and physiologic conditions of perfusion was possible. Addition of chemotherapeutic agent to the perfusate, and inoculation of Walker 256 carcinoma cells into the muscles of the limb, permitted study of the effect of alteration of these controllables on the response to that agent of a well-characterized tumor.

It was established that the effect of perfusion of 4 day old Walker tumors with an alkylating agent, triethylenethiophosphoramide (T.S.P.A.) 4 mgm/kg rat body weight, was to double animal survival time from the day of tumor inoculation. Both grossly and histologically there was suppression of tumor growth for approximately 14 days after perfusion. Subsequent tumor growth progressed in characteristic fashion and metastasized so that the metastatic pattern at death was essentially similar to that in untreated animals.

Studies on variation of perfusate temperature were inconclusive but suggested that the rate of blood flow may be relatively low through tumors which are characterized histologically as being highly vascular. The use of externally applied heat in the form of diathermy was shown to be of no value when heating deep-seated tumors. A high perfusate oxygen tension was shown to potentiate the action of T.S.P.A. on the Walker tumor.

In a controlled, comparative study of systemic administration, intermittent intra-arterial infusion and isolation perfusion chemotherapy, the latter was shown to exert the most profound effect on the tumor as measured in terms of animal survival time. Some variation in the metastatic pattern was seen in this study, but probably reflected the experimental technique used rather than the basic concept of the modality of treatment involved.

These findings are discussed and the future role of both clinical and experimental regional chemotherapy suggested.

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CHAPTER I

"Pour bien comprendre une Science, il faut en connaître l'histoire." (Auguste Comte, 1798-1857).

Until the latter part of the nineteenth century, whatever understanding of cancer existed was based upon studies of morbid anatomy and histology. From such studies stemmed the rationale for treatment either through the complete extirpation of malignant cells by surgical means, or later through their destruction in situ by radiation. The limitation of such methods lay in their failure, to a greater or lesser extent, to cope adequately with one of the outstanding features of malignancy - the propensity of the malignant cell to disseminate. For this reason attention has focussed in recent years upon chemotherapy as a means of curbing malignant growth in a less local and more systemic fashion.

The concept of treating cancer by chemical agents or drugs is perhaps as old as the practice of medicine itself. While the introduction of potassium arsenite, or Fowler's solution, by Lissauer in 1865 for the treatment of leukemia is generally considered the first successful attempt at

cancer chemotherapy (57), it should not be forgotten that chemotherapy of a kind had been practiced by Hippocrates who used surface applications of a variety of caustics or escharotics in the treatment of cancer (55). Through the ensuing centuries continued attempts were made to find a chemical cure for cancer, and such diverse agents as belladonna, aconite, mercury, antimony, copper, red and black salves and a variety of acids and alkalis were tried empirically (56). In 1767 Burrows was forced to conclude that: "Although the Physicians of all nations, from the time of Hippocrates to the present, have, by numberless researches and experiments, made trial of everything in nature, from the most innocent drug, to the most virulent poison, yet the disease still baffles the power of physic...." (23). The desperate empiricism of this approach to cancer chemotherapy was to continue until World War II. At that time man's search for a more noxious gas with which to destroy his enemies led to intensive study of the cytotoxic properties of mustard gas. From this research and the earlier discovery of the affinity of the mustards for rapidly growing tissues (88), came the attempt by Gilman and Philips to treat human lymphosarcoma with nitrogen mustard (47) and with it began the epoch of scientific cancer chemotherapy. It is interesting to note that mustard gas in solution had been used by Adair and Bagg some fifteen years earlier for

surface and interstitial tumor applications (1).

Since 1946 an increasing knowledge of the intermediary metabolic and nucleic acid chemistry of malignant cells has led to the introduction of many new compounds and concepts. The prevailing rational approach to cancer chemotherapy is well illustrated by the introduction of the folic acid antagonists by Farber and his associates in 1948 (40); their search for the sites of action of the compounds increased interest in the metabolism of the purine and pyrimidine base constituents of nucleic acids, and led to deliberate synthesis of chemotherapeutic antimetabolites. Yet it is still commonplace for compounds to be introduced on the basis of what Hall has termed "enlightened empiricism" (57), and many of the more effective alkylating agents, antibiotics and hormones in use to-day were introduced on just such a basis of intelligent guesswork.

To-day a multiplicity of more or less effective chemotherapeutic agents are available, but it is a disappointing truth that even combined with the best efforts of surgery and radiotherapy there remains no certain cure for cancer. One reason for the failure thus far of cancer chemotherapy has been the lack of sufficient selective toxicity in that the anticancer agents affect alike both normal and abnormal rapidly proliferating tissues. Attempts to surmount this hurdle have been both numerous and diverse; while the

biochemist has sought some reaction concerned with cell metabolism or division that is peculiar to neoplastic cells, the surgeon has explored methods of administering presently available anticancer agents whereby their toxicity would be largely confined to the cancerous tissue.

In 1950 Klopp and his associates attempted this by administering nitrogen mustard into a major artery supplying the tumor-bearing region, giving small fractional doses over long periods of time, thereby increasing the total dose with high concentration in the tumor while limiting systemic toxicity (13,14,74,75). In a further attempt to contain the agent within the tumor-bearing region, Klopp also attempted venous occlusion of the part by application of tourniquets. Subsequently many others have used modifications of Klopp's technique (4, 17-19,24,50,63,81,98,108,126,128,135). Klopp, in his original paper (74), outlined a number of principles for the further development of intra-arterial injection, including (1) intravenous injection of a non-toxic antagonist at the time of intra-arterial injection of the chemotherapeutic agent to reduce systemic toxicity, and (2) use of a heart-lung mechanical pump to maintain the circulation of an isolated tumor-bearing area. Sullivan and his colleagues were the first to explore Klopp's suggestion of using an antagonist to decrease the systemic toxicity. They employed intermittent intramuscular injections of Citrovorum Factor (Leucovorin) to counteract the toxic systemic effects of Methotrexate which

was infused continuously for six to ten days (35,129,131,132). This technique is gaining in popularity (25,60,134), and other chemotherapeutic agents are being tried in this manner (39,82,130,133).

More dramatic however was the introduction by Creech, Ryan and their co-workers (110) of a method for intravascular perfusion utilizing an extracorporeal circuit incorporating a pump-oxygenator, in accordance with Klopp's earlier suggestion. They treated their first patient by perfusion in June, 1957. Briefly the technique requires that a major artery and vein supplying the tumor-bearing region be cannulated, and the region isolated insofar as is possible from the general body vasculature by occluding collateral vessels and, if possible, by application of a tourniquet. The extracorporeal part of the circuit includes a blood reservoir and oxygenator from which blood is pumped into the arterial cannula. The venous return from the isolated region is either allowed to flow out by gravity or may be extracted by another pump which also serves to return the blood to the reservoir.

The attractive feature of this technique is the degree of isolation that might be achieved. Theoretically it should be possible to localize the effects of the chemotherapeutic agent and protect susceptible normal tissues such as the bone-marrow and gastro-intestinal tract, thus permitting the use of agents which are not fixed rapidly or do not act

quickly. Further, the only limiting factor with regard to dosage of the chemotherapeutic agent would be the local tissue tolerance, so that concentrations of the agent in the tumor region could greatly exceed the maximum permissible concentration achieved by systemic administration. It is also possible at the conclusion of perfusion to remove residual drug by flushing out the perfused area with dextran. In practice such a degree of isolation is seldom realized (62,89,93,118,121) and the amount of agent administered is adjusted according to the completeness of isolation, the size of the perfused area, the duration of action of the chemotherapeutic agent, and whether or not the patient has received prior chemical therapy or irradiation (33).

In addition to providing a means of limiting systemic toxicity the technique permits controlled alteration of the physiologic and biochemical environment of the tumor (9). Thus it should be possible to alter local physicochemical conditions in the perfused tissues in such a manner as to attain the optimum environment for the effective anticancer activity of the agent employed. For example, the distribution of perfusate in the perfused tissues might be expected to depend upon flow-rate and perfusion pressure (11,33), and the use of a suitable neurogenic blocking-agent might decrease vascular spasm and increase blood flow to small vessels (9). Furthermore, in common with other chemical reactions, the activity of any chemotherapeutic agent would presumably be greatest under certain conditions

of temperature (140), pH (138) and oxygenation (49,87). Since many chemotherapeutic agents are highly unstable in blood under physiologic conditions (93) and since the amount of leakage from the perfused tissues into the systemic circulation increases, inter alia, with time (121) the duration of perfusion would also assume importance.

In the absence of what Creech has described as "an agent as effective as aspirin and as free from toxic side-effects" (33), isolation perfusion of regionally confined neoplasms offered an attractive approach to cancer chemotherapy. It soon became evident, however, that there was wide variation in the response of a given lesion to a given agent. For example, while rapid resolution of metastatic melanoma might follow perfusion with phenylalanine mustard in one case, there might be no response at all in another (33). This has been the experience of many of the surgeons who have employed this technique, including Creech and his associates (30-34, 83,84,106,110), Stehlin (119-123), Woodhall (139,140), Shingleton (116,118), Ausman (9,10) and many others (28,61,66-68,70,76,91,94,141).

This variation in response, coupled with attempts to define the ideal conditions for effective chemotherapy, has stimulated extensive research. Most related experimental work has been performed on the dog (5,7-9,15,16,20,26,58,62, 64,69,71,73,89,99,102,103,105,109,111,112,114,116,117) although Economou and his associates (21) and Clarkson (28) used the rabbit. The dog is satisfactory for studying the

mechanics of perfusion and for assessing expected normal tissue tolerance to high concentrations of anticancer agents under the conditions of perfusion, but the lack of naturally occurring or transplantable tumors in dogs is inconvenient. For this reason, and since uniformity in size, sex, age and strain are readily attainable in rats, a technique for isolation perfusion of the rat hind limb has been developed. It is possible to transplant the Walker 256 carcinoma into the limb muscles of Sprague-Dawley rats, thus permitting a study of the many variables associated with perfusion in terms of the response of a well-characterized tumor. This technique also appears to offer possibilities as a preliminary screening procedure for anticancer agents since it permits the use of much higher concentrations than would be possible by systemic means; failure of the agent at such concentrations would deem success unlikely at more usual systemic levels.

This thesis will describe the technique that has been evolved, its use in a comparative study of several modalities of cancer chemotherapy, and finally the histopathologic changes observed in the Walker tumor perfused with an alkylating agent.

CHAPTER II

THE TECHNIQUE FOR ISOLATION PERFUSION OF THE RAT HIND LIMB

APPARATUS

Blood reservoir, oxygenator and debubbler (Fig. 1, A).

A disposable miniature bubble oxygenator was constructed from a 25 inch length of polyethylene tubing having an internal diameter of 1/4 inch. This was held by clamps in the position illustrated. Fresh heparinized blood, obtained from donor rats (vide infra), was poured into the first descending limb and carried up the ascending limb of the oxygenator by oxygen introduced at 1-2 liters/min through two 27 gauge needles. Excess oxygen escaped through a vent (13 gauge needle) at the commencement of the second descending limb. This limb was siliconized using a liquid silicone preparation and functioned as a debubbler and reservoir for oxygenated blood.

Pump (Fig. 1, B).

A Sigmamotor finger-pump (Model T.M. 11 - Middleport, N.Y.) was used and proved very reliable, the vernier control allowing easy calibration and good reproducibility of the desired flow-rate. A 5 inch length of firm rubber tubing having an internal diameter of 3/64 inch and an outside diameter of 1/8 inch was used between the finger and pressure plate of the pump. It was held in

place by two collars of surgical tape placed around the tubing where it entered and left the pump housing. One end of the tubing was connected through a tapered, polished stainless steel connector to the oxygenator, the other through a simple rubber connector to the heat exchanger.

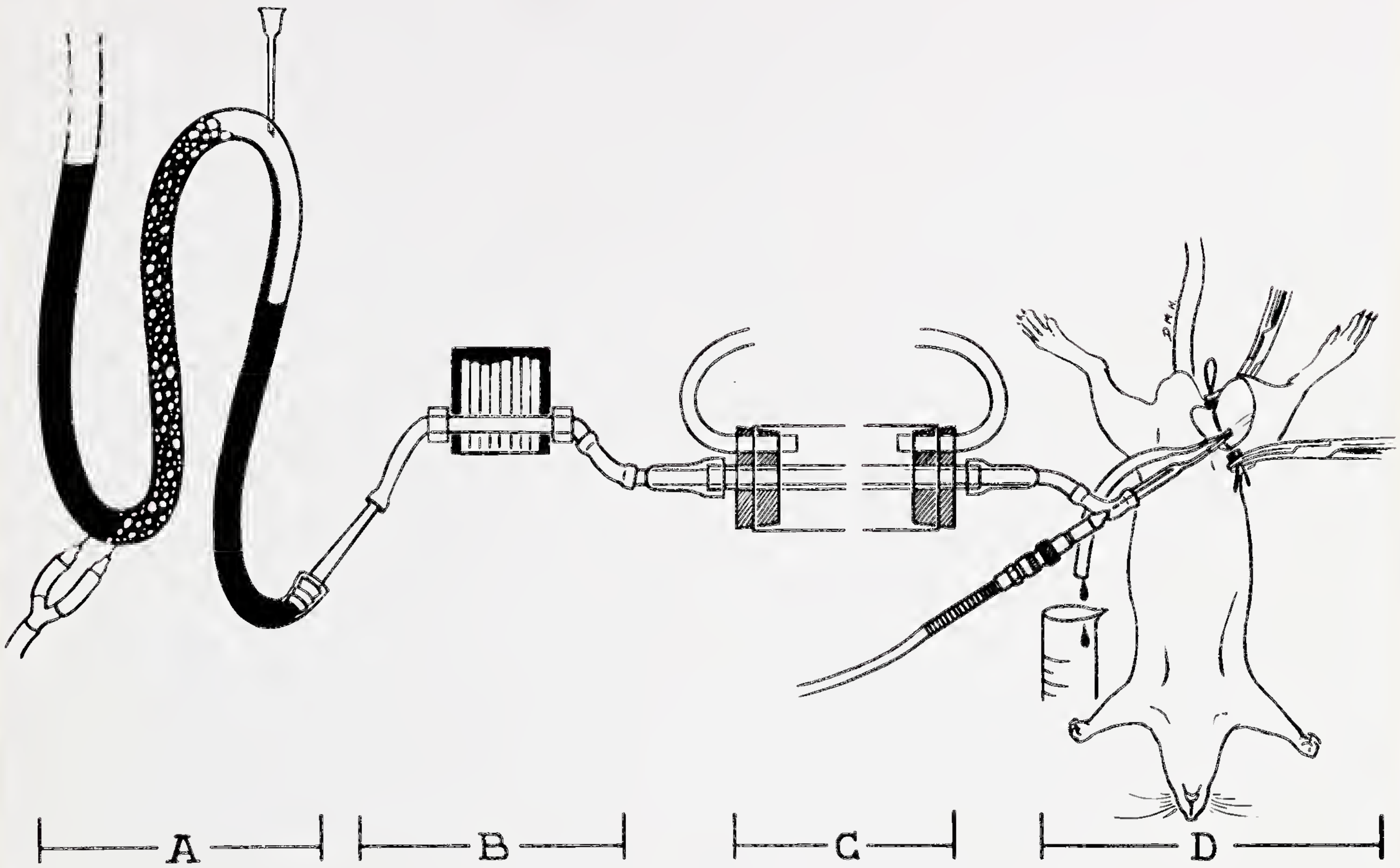
Heat exchanger (Fig. 1, C).

This was constructed from an 8 inch length of polyethylene tubing, 1 1/2 inches in diameter, and closed at either end by rubber stoppers, each stopper being pierced by two holes. A 9 inch length of a hard, clear, plastic tubing having an internal diameter of 3/16 inch was then passed through one hole of each stopper such that a water-tight fit was obtained. The other hole in each stopper was connected to a circulating water pump (Model K1 - Gormann Rupp Industries, Belleville, Ohio) which circulated water at a predetermined temperature. In use, a length of PE 280 polyethylene tubing (Clay-Adams, New York) was passed through the narrower plastic tube of the water-jacket and connected by short rubber connectors to the pump and to the arterial cannula. This heat exchanger was designed to permit rapid changing of the perfusion apparatus; it was only necessary to replace the PE 280 tubing between perfusions, and prolonged cleaning and sterilizing operations were thus obviated.

Cannulae (Fig. 1, D, and Fig. 2)

The arterial and venous cannulae were made from

FIGURE 1



Diagrammatic representation of perfusion apparatus.

Compare with Fig. 4.

A. Blood reservoir, oxygenator and debubbler.

B. Sigmamotor finger-pump.

C. Heat exchanger.

D. Femoral artery and vein cannulated. Thermistor in arterial cannula. Tourniquet in position deep to femoral vessels.

PE 280 polyethylene tubing. A short length (approx. 15 cm) was softened at its mid-point by heating over a small flame and drawn out to a fine taper. It was allowed to cool and then cut into two halves; the points of the two cannulae thus formed were trimmed to a slight bevel. The arterial cannula was kept short to minimize heat loss between the heat exchanger and the perfused limb. The length of the venous cannula was unimportant; it was connected to a further length of PE 280 tubing which emptied into a graduated collecting vessel to measure venous return.

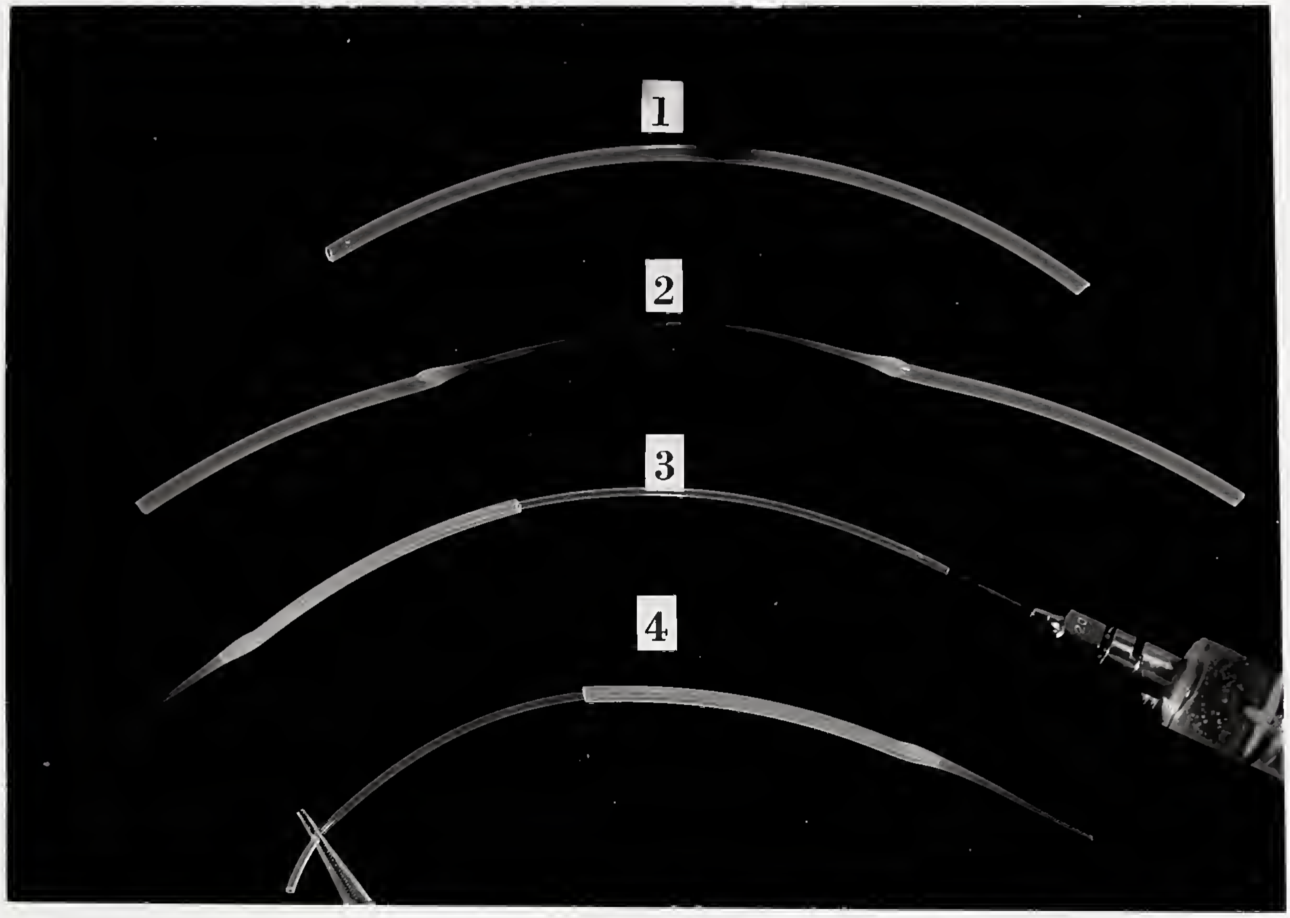
Temperature monitoring (Fig. 1, D).

Continuous monitoring of the arterial line temperature was achieved using a Thermistor Probe ("500" Series Hypodermic Probes, Yellow Springs Instrument Co., Ohio.) This was inserted through the rubber connector between the heat exchanger and the arterial cannula.

Injection of chemotherapeutic agents.

In clinical perfusions it has been customary to inject drugs into the arterial line at a pre-pump level to avoid streaming and to ensure adequate mixing (83). Because of the low flow rates used in rat perfusions this was considered unnecessary, and in order to ensure that all the drug injected reached the perfused limb the drug was injected between the pump and the arterial cannula. Since the injection of a cold fluid into the arterial cannula was

FIGURE 2



Stages in the preparation of cannulae.

(1) 15 cm length of PE 280 polyethylene tubing.

(2) Tubing has been softened by heating, and drawn out.

(3) Cannula with inner PE 90 tubing in place and filled with heparin.

(4) Syringe has been removed and PE 90 tubing clamped.

Note that clamp is not applied directly on main cannula.

undesirable, the site chosen for injection was the rubber connector between the pump and the heat exchanger.

ANIMALS.

Male Sprague-Dawley rats weighing from 250 to 350 grams were used. These were obtained by two suppliers: Northwest Rodent Co., Pullman, Washington, and National Laboratory Breeding Co., Edmonton, Alberta. All rats were fed a standard laboratory chow with water ad libitum, and were kept in individual cages after surgery. Rats which did not appear in good health were destroyed where this could not be attributed to the experimental procedure. During the course of these studies occasional outbreaks of a viral pneumonia, which is endemic in North American rats, were experienced; when this occurred the entire rat colony was destroyed, rooms and equipment were sterilized and fresh healthy rats imported.

Tumor and method of transplant.

The Walker 256 carcinoma was used because of its ease of transmission and reported lack of regression (43). This tumor, first observed by Walker in 1928, was described in morphological detail by Earle in 1935 (36). It has been variously termed a carcinosarcoma, sarcoma or simply just a tumor, but recent electron microscopic, histologic and histochemical studies by Fisher and Fisher (43) were in agreement with Earle's earlier opinion that the designation of this tumor as a carcinoma was most accurate

(see Chapter 5.)

The tumor was maintained by transplanting every seventh day into new rats. An untreated tumor rat was anesthetized with intra-peritoneal sodium pentobarbital, 40 mgm/kg body weight, and the tumor-bearing leg shaved and prepared with 2 1/2%tincture of iodine. Using aseptic technique, a long groin incision was made and the skin and subcutaneous tissues reflected off the medial and posterior aspects of the thigh muscles. These muscles were incised down to the tumor and the tumor opened to allow the necrotic tissue in the center of the tumor to escape. The healthy peripheral tumor tissue was dissected out in piecemeal fashion and placed temporarily in a Petri dish containing cold sterile isotonic saline. Approximately 2 grams of this tissue were then placed in a Pyrex tissue grinder with 10 cc of sterile saline, and a homogenate prepared. 0.15 cc of this homogenate was inoculated through a 20 gauge needle into the posterior muscle mass of the hind limb of the recipient rat. The recipient animals were unanesthetized but were held firmly by an assistant. Cell counts were not performed (101), nor was any attempt made to age the cells (22) since 100% "takes" were sought. Using this technique 95-100% "takes" were regularly achieved.

Method of obtaining blood from donor rats.

Blood to fill the oxygenator and reservoir was obtained from healthy, non-tumor-bearing rats. The donor rats were anesthetized with ether so that barbiturate in the perfused blood would not endanger the perfused rat should any appreciable leak into the systemic blood supply occur. The anterior abdominal wall was opened through a long mid-line incision, the abdominal viscera reflected laterally and the aorta exposed. The aorta was clamped just above the bifurcation, and blood aspirated by direct needle puncture (19 or 20 gauge needle) into a syringe containing 10 mgm heparin sodium; between 12 and 15 cc of blood could be aspirated from a 300 gram rat in this manner. When deoxygenated blood was required, direct needle puncture of the right ventricle was performed. This was most easily accomplished by opening the abdomen and incising the tendinous part of the diaphragm; this prevented the very considerable blood loss that occurred when the rib cage and thoracic muscles were cut across. Using the trans-abdominal approach it was not difficult to aspirate 10 to 12 cc of blood from the right ventricle.

Preparation of rats for perfusion: operative technique.

Perfusion was only possible within the first ten days of tumor growth; thereafter the size of the tumor made cannulation of the femoral vessels technically difficult. The rats were anesthetized with intraperitoneal sodium pentobarbital, 35 mgm/kg body weight. This was a smaller

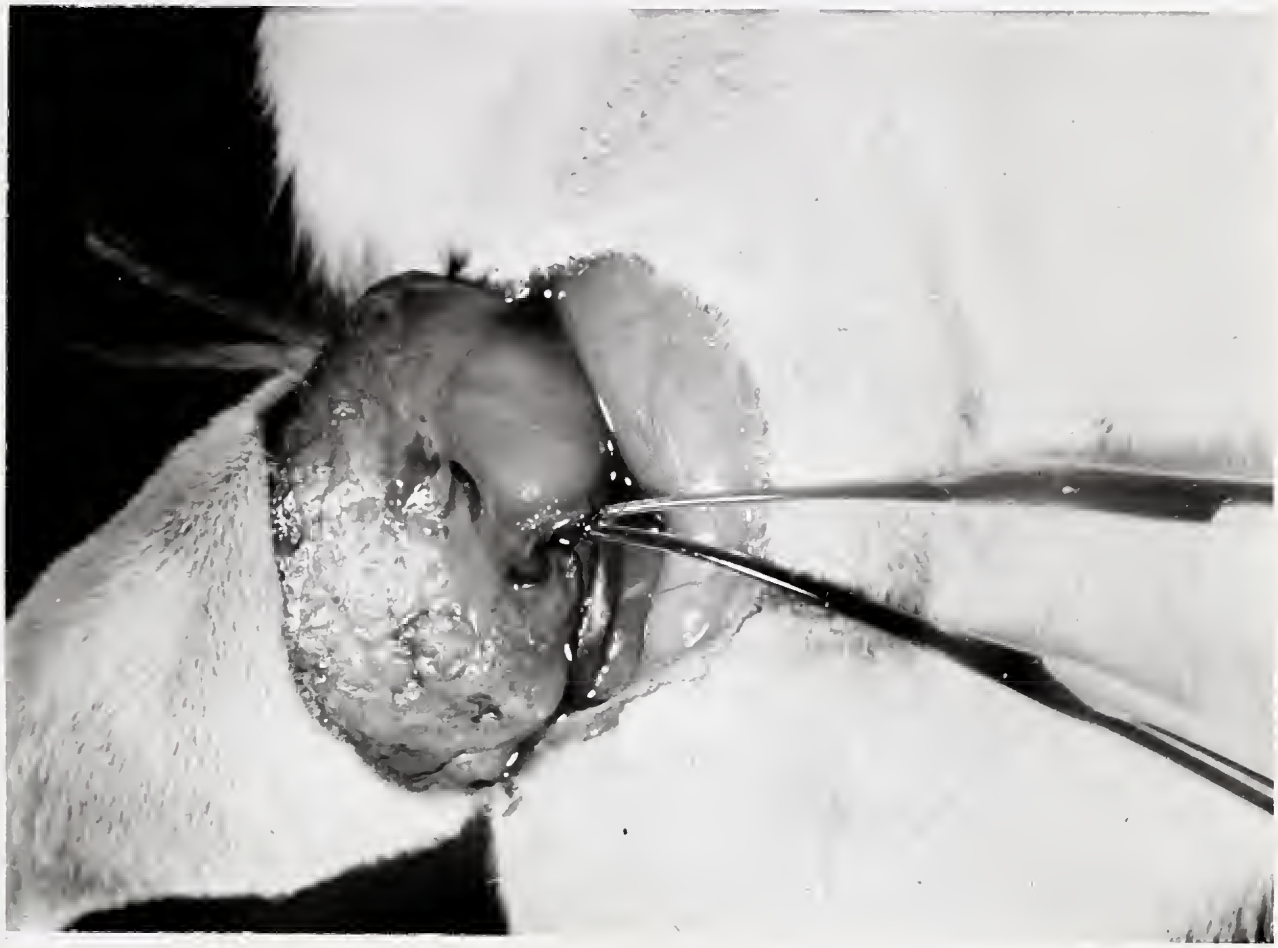
dose than that given to the donor rats for tumor transplant, but was sufficient to keep the rat anesthetized for 45 to 60 minutes. The tumor-bearing limb was prepared by shaving and painting with 2 1/2% tincture of iodine, and the rat was then taped in a supine position on a thin stainless steel plate measuring 20 X 30 cm.

A long groin incision was made parallel to the inguinal ligament (Fig. 3). A layer of thick subcutaneous fat that filled the cleft of the groin was separated from the abdominal muscles and reflected distally. Dissection was then carried down to the femoral vessels which were easily recognized as they entered the limb beneath the inguinal ligament. The femoral artery and vein were then dissected free from each other and from the femoral nerve; they were cleaned of all areolar tissue from the inguinal ligament as far distally as their first major branch, a 7-8 mm length of vessel thus being available for cannulation.

The cannulae were prepared by threading a 15 cm length of PE 90 tubing along the lumen of the cannula as far into the tapered portion as it would go. This tubing and the remaining part of the tip of the cannula were then filled with a dilute heparin solution (10 mgm heparin in 10 cc sterile isotonic saline), the syringe containing the heparin being left attached to the tubing (Fig. 2, (3)).

Two ligatures of 4-0 black silk were then placed about the femoral artery, the proximal ligature being placed

FIGURE 3

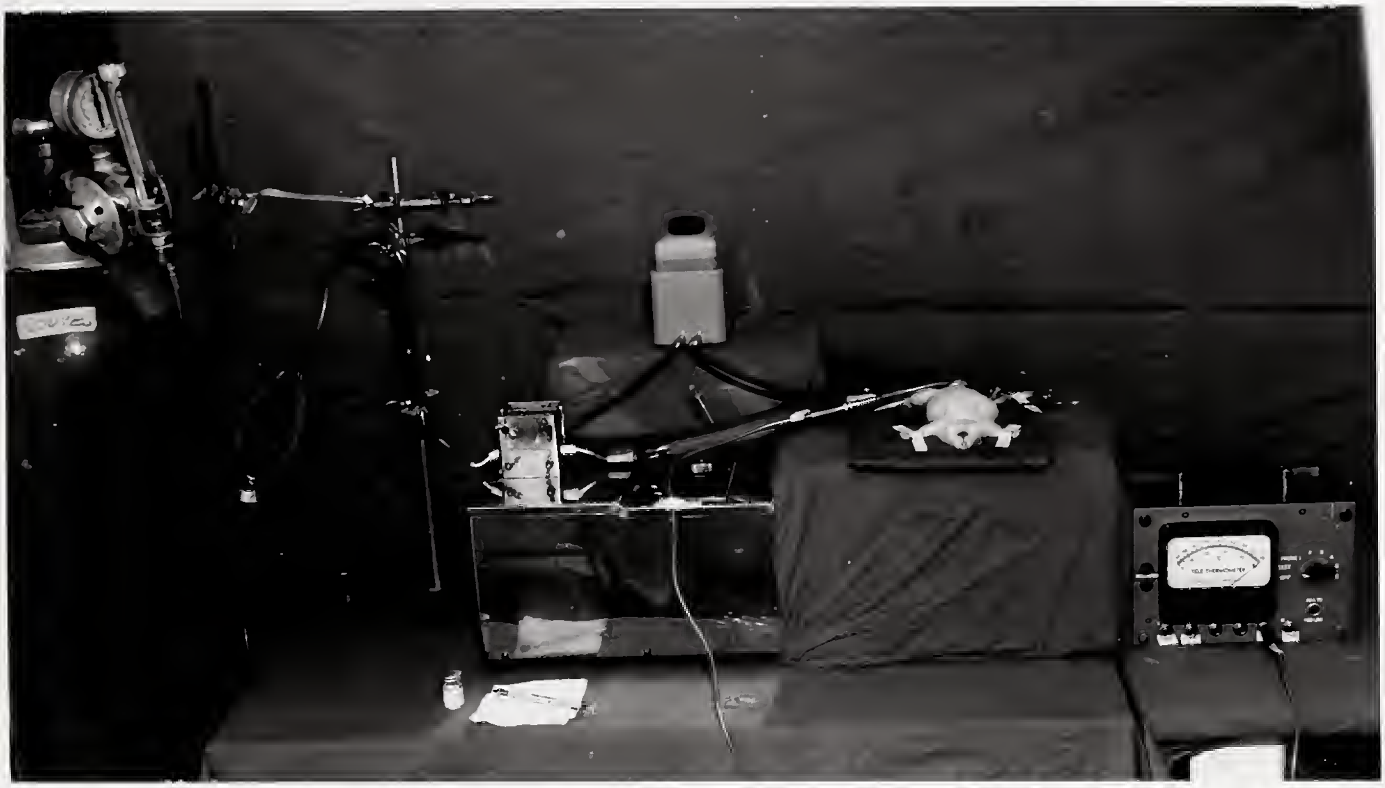


Photograph of incision with cannulae and tourniquet in place. The arterial cannula is uppermost in this picture. The tourniquet has been tied with a knot to prevent reflections from instruments in the photograph.

as close to the inguinal ligament as possible and then tied; its ends were left long and held by a hemostat. The first knot of the distal ligature was made but not tied, and the ligature positioned as far distally as possible; its ends were similarly held by a hemostat. By means of the hemostats holding the ends of the ligatures, slight tension was applied to the vessel and a small transverse incision made in the arterial wall about 5 mm below the inguinal ligament. This incision was most easily made with a small pair of sharp-pointed dissecting scissors. The cannula was then inserted and threaded distally for a distance of about 4 mm. and the distal ligature tied firmly over the cannula without occluding the lumen. If the cannulation was successful the cannula filled rapidly with blood. The rat was then heparinized through the arterial cannula by injecting 1 cc (1 mgm) of the dilute heparin solution. Finally the PE 90 tubing was clamped beyond the cannula and the syringe removed (Fig. 2, (4)). The femoral vein was cannulated in an identical manner.

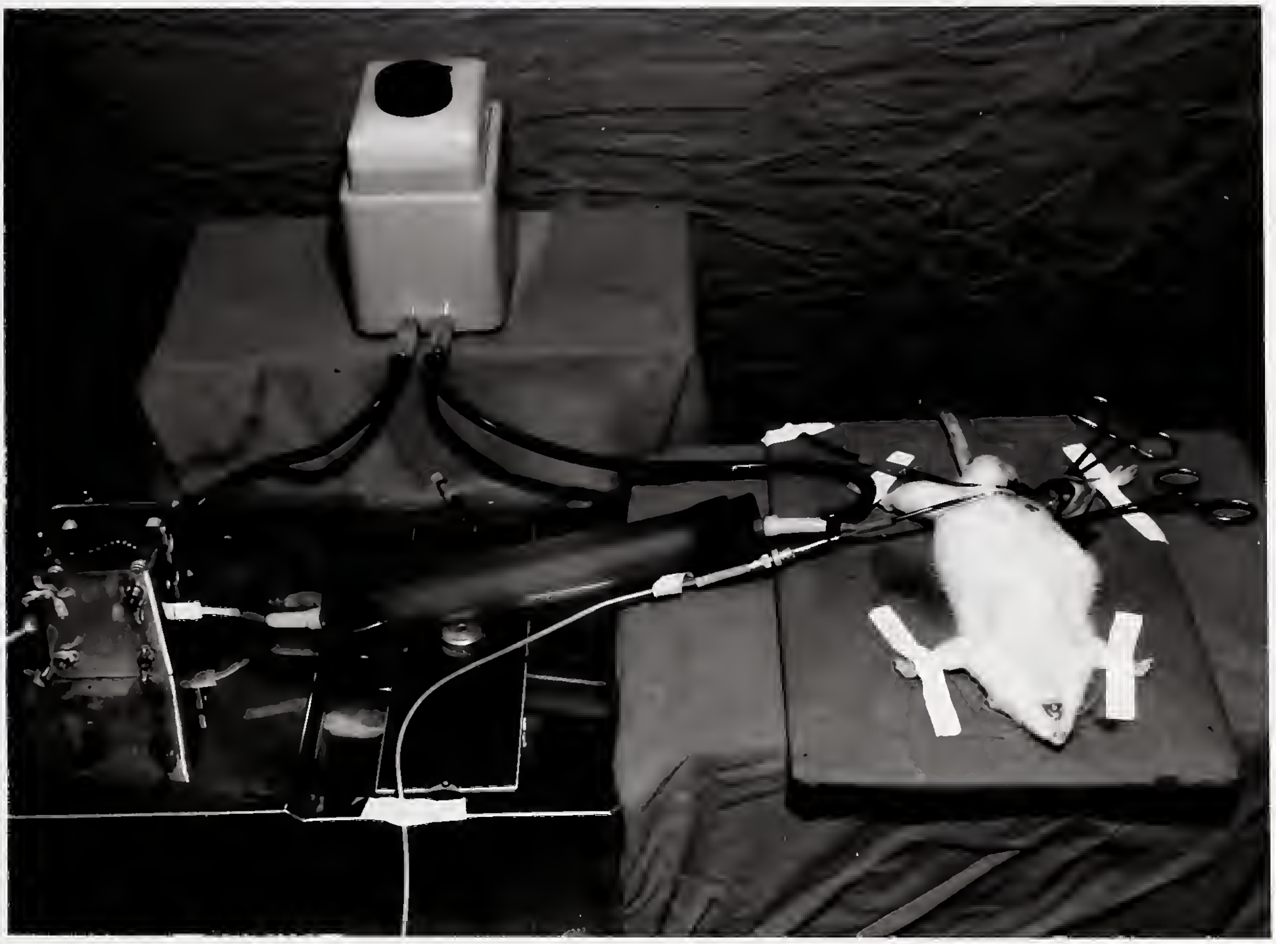
When cannulation of both vessels was complete a tourniquet, consisting of an ordinary rubber band about 10 cm in length and 2 mm in diameter, was applied around the limb passing deep to the cannulated femoral vessels and through separate stab wounds at either end of the main incision. It was anchored to the inguinal ligament with a single 000 silk suture to prevent it from slipping down the limb and

FIGURE 4



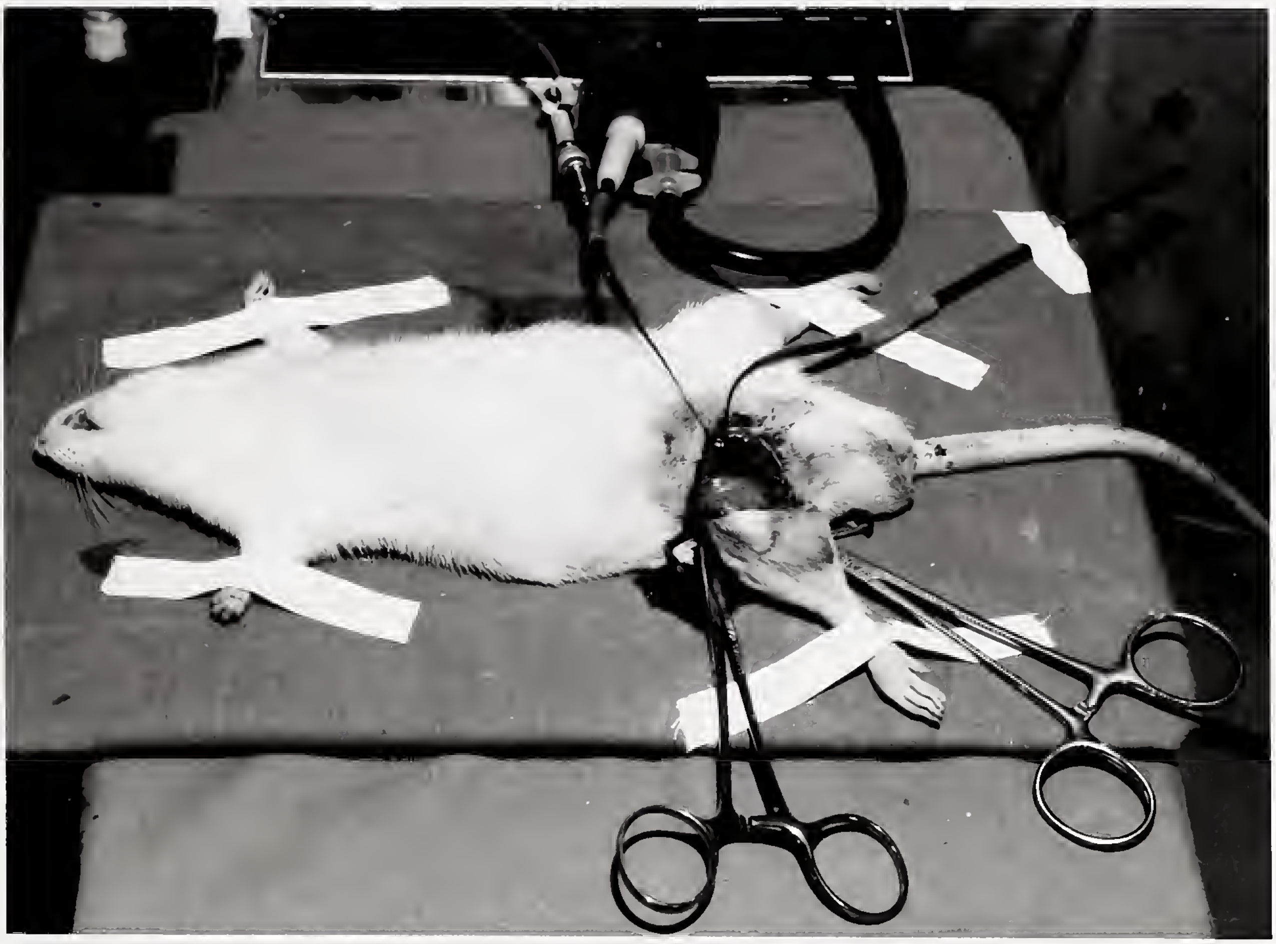
Photograph of perfusion in progress. This figure should be compared with Fig. 1.

FIGURE 5



Photograph to illustrate the position of the rat relative to the perfusion apparatus and the position of the thermistor in the arterial cannula.

FIGURE 6



Photograph showing the clamps used to tighten the tourniquet and the method of gravity venous drainage.

obstructing the cannulae. The tourniquet was left loose until perfusion was begun. The incision, with the cannulae and tourniquet in place, is shown in Fig. 3.

PERFUSION.

The combined blood reservoir, oxygenator and debubbler was filled with fresh heparinized blood obtained from donor rats in the manner described. The pump was started to fill the perfusion line. The inner PE 90 tubes were removed from the cannulae and the cannulae allowed to fill with blood; the rat was then connected to the perfusion circuit, the tourniquet tightened by applying small forceps on either side of the limb, and perfusion commenced (Figs. 4, 5 and 6). In the standard perfusion a flow rate of 0.5 cc/min was maintained and perfusion continued for 15 minutes. The temperature in the arterial cannula was maintained at 37°C. The chemotherapeutic agent was usually administered in equally divided doses at 0, 5 and 10 minutes.

At the conclusion of perfusion the pump was stopped, the tourniquet released and the cannulae removed; the femoral vessels were ligated and the wound closed in layers with interrupted 000 silk sutures. The rats were then returned to individual cages to recover from the anesthetic. Disposable parts of the apparatus (plastic and rubber) were replaced after each perfusion; other parts were cleaned thoroughly with water, aqueous zephiran and sterile isotonic saline.

Comment.

Several factors have emerged as being of great importance for successful perfusion. While the use of aseptic technique was mandatory, of equal importance was absolute cleanliness of all equipment since the smallest particles of foreign material acted as emboli and resulted in gangrene of the perfused limb. Similarly silicone embolization occurred if excess silicone was left in the debubbler. In this context it was found that small air bubbles were not important; they were often observed to pass through the limb, and in the pressure studies reported in Chapter III air was deliberately introduced into the arterial cannula without ill effect.

The venous return was the best indicator of the adequacy of the perfusion and required constant observation. If difficulty was encountered in establishing a good venous return (greater than 0.4 cc/min) the procedure was abandoned since gangrene of the limb almost inevitably resulted. With experience this complication became very rare. Over-perfusion was prevented by making connections which were a good fit but not excessively tight; in the event of incipient over-perfusion the pressure build-up caused the connections to "blow".

The technique of cannulation was not difficult and was easily performed under direct vision. When large numbers of perfusions were necessary it was simplest to use three operators; the first anesthetized and cannulated rats; the second managed the perfusion and recorded data; the

third was responsible for obtaining blood from donor rats and for closing the wound in the perfused rats. In this way it was possible to complete twenty perfusions in an eight hour period using a single pump.

CHAPTER III

THE STANDARD PERFUSION. STUDIES ON THE PHYSIOLOGIC AND BIOCHEMICAL CONDITIONS OF PERFUSION.

In the previous chapter the technique for isolation perfusion of the rat hind limb was described, and reference made to the "standard perfusion". Many of the conditions of the standard perfusion were arrived at early in this work on the basis of small pilot studies (some of which have been repeated subsequent to refinements in technique), or on largely theoretical grounds; these are described in this chapter. Having defined the standard perfusion, the response of the Walker tumor to perfusion under standard conditions is described. Finally two studies are described which illustrate the use of the technique of isolation perfusion to evaluate the effect of alteration of tumor environment on the efficacy of the antitumor activity of an alkylating agent. Because this chapter embraces a heterogeneous collection of data, each study is discussed, when appropriate, as it is described; this obviates a voluminous discussion at the end of the chapter and the necessity of constant referral back to the relevant subsection.

PART I. THE STANDARD PERFUSION

Chemotherapeutic Agent and Dose.

Because in this work our interest lay with the technique of administering chemotherapeutic agents, the alkylating agent triethylenethiophosphoramide was used throughout to provide easy comparison of data. Short trials were made using other alkylating agents (nitrogen mustard (HN2), phenylalanine mustard (PAM) and cyclophosphamide) and one antibiotic (actinomycin D) to determine the applicability of the technique as a screening test for anticancer agents; these studies were small and since they were not directly related to the technique of perfusion are not reported here.

Triethylenethiophosphoramide (thioTEPA, T.S.P.A.) is a polyfunctional alkylating agent, meaning that it has more than one reactive center. It is a triethyleneimine which was deliberately synthesized after it became clear that the mode of action of nitrogen mustard involved an intermediate reactive ethyleneimine stage. The ethyleneimine groups are capable of interacting with nucleophilic groups such as amino and sulfhydryl groups in biologic materials, and with the anions of organic acids (57). The groups which are capable of being alkylated at physiological pH will depend on the dissociation constant of the particular group. It has been suggested that alkylating agents interfere with the hydrogen bonds linking the two nucleotide

chains of deoxyribose nucleic acid (DNA) through their action on the amine groups of adenine and cytosine, both of which are weak and dissociated at physiologic pH (57). However the final answer to the question of where and how alkylation occurs remains to be decided (100).

In the standard perfusion T.S.P.A. was used at a dose level of 4 mgm/kg rat body weight. This dose was selected because pilot studies had shown that it provided a satisfactory tumor response without causing any appreciable normal tissue damage. Larger doses, while enhancing tumor response, also increased the incidence of limb damage (44). Furthermore this dose was well tolerated by the rat when given systemically, and could thus be used with safety in a comparative study of perfusion and systemic chemotherapy (see Chapter 4). Since the volume of the rat hind limb approximates 12 1/2% of the total blood volume, then the local tissue concentration of T.S.P.A. during perfusion was approximately 8 times greater than that achieved by systemic administration of the same dose.

Tumor Age.

In earlier studies, Francis et al., (45) had observed that it was not until the 4th. day of tumor growth that any really significant effect of perfusion was seen. After the 10th. day of tumor growth the size of the tumor made cannulation technically difficult. It is also worthy

of note that before the 4th. day it was difficult to judge in any reliable way the presence or absence of a successful tumor "take". For these reasons, together with a touch of empiricism, 4 day old tumors were usually chosen for study and came to be regarded as standard.

Flow Rate and Perfusion Pressure.

An attempt to assess the normal blood flow rate through the rat hind limb was made by cannulating the femoral veins of 5 limbs. In 2 of these limbs a tourniquet was placed around the limb, beneath the femoral vessels, in the manner used for isolation perfusion. It was found that in the absence of a tourniquet the venous outflow collected over two consecutive 2 minute periods averaged 0.46 cc/min. With the tourniquet in place the outflow averaged 0.47 cc/min (53). On the basis of these findings a flow rate of 0.5 cc/min was adopted for perfusion and proved highly satisfactory. In a small series it was shown that flow rates greater than 1.0 cc/min were unsuccessful because of obvious over-perfusion; flow rates above 3.0 cc/min uniformly resulted in destroyed limbs and the immediate tumor response was no greater than with a physiologic flow (44).

Early attempts to monitor the arterial line pressure during perfusion were largely unsatisfactory because of difficulties with cannulation which resulted in sudden and uncontrollable elevations of pressure (53). A linear relationship was however demonstrated between arterial line

pressure and flow rates over the range of 0.1 to 1.2 cc/min, in accordance with the well-known Poiseuille-Hagen Law. Recently, and with improved facility of cannulation, the arterial line pressure was monitored in 8 successive perfusions.

Method. Standard perfusions were performed, maintaining a flow rate of 0.5 cc/min for 15 minutes. T.S.P.A., 4 mgm/kg was administered in the usual way. The temperature in the arterial line was 37°C. A continuous pressure trace was obtained using a Statham strain gauge (Statham Instruments, Inc., Puerto Rico) and a Gilson multichannel recorder (Gilson Medical Electronics, Middleton, Wis.). End-pressures were recorded through a short 20 gauge needle inserted into the rubber connector between the heat exchanger and the arterial cannula. In addition pressures were recorded in two rats from the opposite femoral artery as a measurement of normal rat systemic blood pressure.

During two recordings the effect of kinking the venous cannula on the arterial line pressure was observed. In a further two cases 0.5 cc of air was deliberately injected into the arterial cannula and the pressure recorded while the resulting air bubbles passed through the limb vasculature.

Results. In all 8 perfusions a remarkably smooth and consistent pressure trace was obtained. A typical example is shown in Fig. 7, A. At the onset of perfusion the arterial line pressure measured about 100 mmHg. Subsequently a slow drop in pressure occurred, so that by the end of a 15 minute

perfusion the arterial line pressure was steady at about 70 mmHg. No sudden alterations in pressure were experienced. Fig. 7, B illustrates the effect of air bubbles in the arterial line; a slight rise in pressure occurred as air entered the limb, but this soon returned to normal levels and there was no long-term effect on pressure. Fig. 7, C shows the sudden rise in arterial line pressure which occurred when the venous cannula was momentarily kinked. The normal rat systemic pressure is illustrated by Fig. 7, D; a mean value of about 100 mmHg was recorded.

Discussion. The optimum rate of flow of blood into an area during regional perfusion is not known (115). However, other things being equal, flow rate will determine perfusion pressure, and since circulatory leak from the perfusion circuit to the systemic circulation becomes excessive at perfusion pressures greater than the systemic pressure (33), it is customary to adjust the flow rate so that the perfusion pressure remains some 20 mmHg below systemic pressure (28,68, 91). Boyland et al., (20) have shown that in the dog limb a wide range of flow rates are accommodated by the limb vascular resistance, but no gross difference in uptake of drug was observed over a wide range of flow rates. They suggest that the use of drugs such as papaverine and histamine, which have been advocated to overcome vascular spasm (68) and to increase capillary permeability (91) respectively, is inadvisable as they open up shunts and might cause a

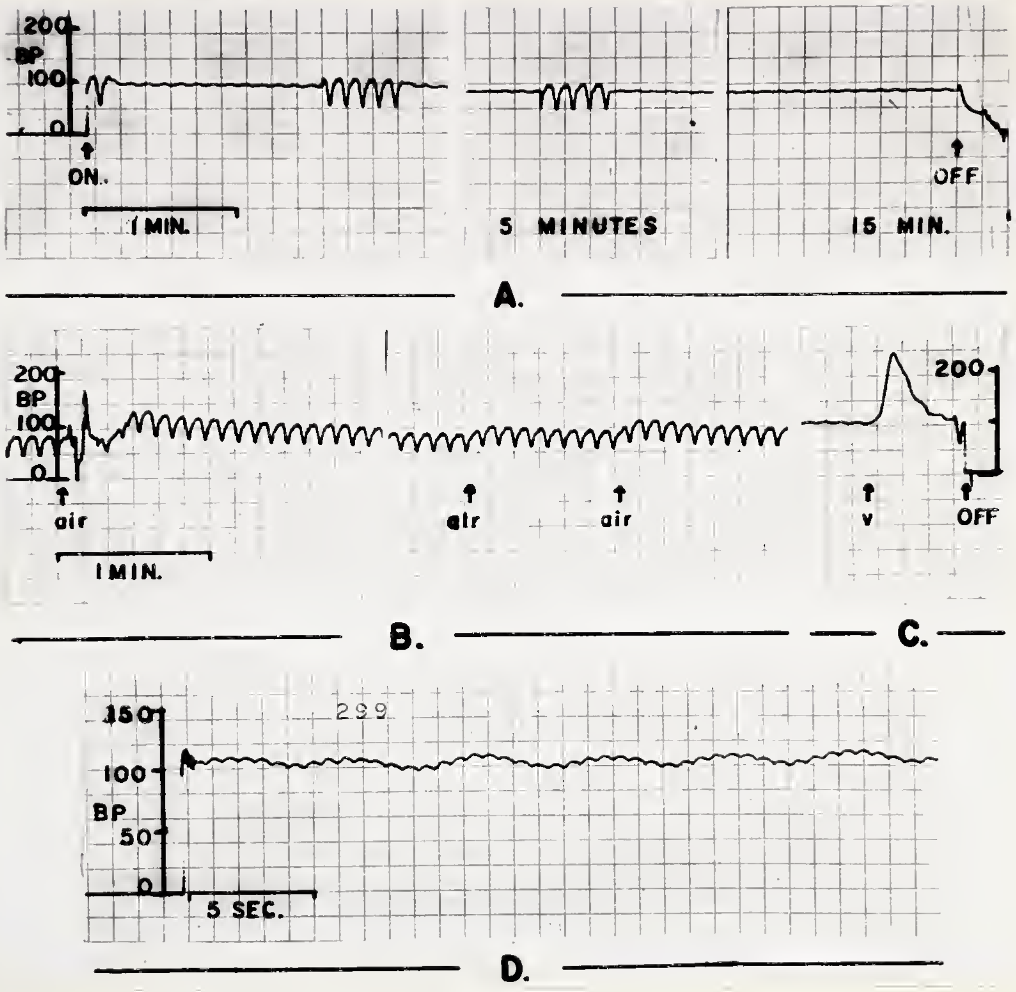
partial bypass of the tumor-bearing region.

In the rat hind limb perfusion, the flow rate of 0.5 cc/min that was adopted as standard has proved in the course of many hundreds of perfusions to be adequate in maintaining a healthy, viable limb, and achieved the desired aim of providing a perfusion pressure slightly below systemic pressure. The rapid increase in pressure associated with venous outflow obstruction will be shown later to result in excessive circulatory leak between the perfusion circuit and the systemic circuit. Since the occasional introduction of air bubbles into the perfusion circuit has been observed, it was comforting to find that they produced only small and transient elevations of pressure.

Duration of Perfusion.

The standard perfusion was continued for 15 minutes, drug being injected at 0, 5 and 10 minutes. Occasionally perfusions were prolonged for periods up to one hour, but since recirculation was not carried out then only sufficient time was needed to allow a single passage of the drug-carrying perfusate volume through the tumor region. Furthermore, prolonged perfusion was not economical because much larger volumes of donor blood were required; and since an adequate tumor response for experimental purposes was obtained in 15 minutes, and pilot studies showed little or no improvement in survival time with longer perfusions, there was no apparent

FIGURE 7



Pressure traces recorded from the arterial cannula during standard perfusions. Pressure calibration and time intervals shown.

A. Typical normal trace. Recording shows mean pressure and, at intervals, the pressure pulse produced by the Sigmamotor pump.

B. Effect of introduction of 0.5 cc air. Subsequent markers indicating "air" denote the passage of residual small bubbles through the limb.

C. Effect of venous outflow obstruction. Venous cannula kinked momentarily at "v".

D. Normal rat systemic pressure recorded from femoral artery.

justification for prolonged perfusion.

Perfusate Temperature.

An extensive study of perfusate temperature using the rat hind limb perfusion technique is described in Part 4 of this chapter. Suffice it at this point to say that in the standard perfusion the temperature of the perfusate in the arterial cannula was maintained at 37°C. This necessitated a heat exchanger temperature of 40°C.

Oxygen Saturation of the Perfusate.

This subject also is discussed in detail in Part 4 of this chapter. In the standard perfusion a bubble oxygenator was employed which maintained an oxygen saturation consistently above 95%.

pH of the Perfusate.

Since the use of a bubble oxygenator has been shown to cause a rise in pH to levels as high as 8.10 (85), it was of interest to measure pH in the standard perfusion.

Method. pH was measured during 5 standard perfusions using 100% O_2 in the bubble oxygenator. Samples of the perfusate were taken at 3 points:

(1) Immediately after the perfusate had left the oxygenator.

(2) From the arterial cannula; this sample contained admixed T.S.P.A. and was heated to 37°C.

(3) From the venous cannula.

pH of the samples was determined using a micro-pH meter (pH Meter 22, Radiometer, Copenhagen).

Results. The pH values recorded are shown in Table 1. It was evident that while the perfusate leaving the oxygenator tended towards alkalinity, this effect was offset by addition of T.S.P.A. so that the perfusate entering the limb had a pH within physiologic limits. The venous pH was still further reduced, presumably as the result of normal metabolism within the tissues of the perfused limb.

Discussion. Relatively little has been written concerning the role of pH in isolation perfusion chemotherapy. There is some evidence that nitrogen mustard at a pH of 2 produces less toxic reactions and has a better therapeutic index than at normal blood pH levels (138), and accordingly there may be an indication for using a perfusing medium with a low pH, as might be produced by the use of greater concentrations of carbon dioxide in the perfusate. Ausman (6,9) has shown an improved utilization of nitrogen mustard in the perfused dog limb using 70% O_2 and 30% CO_2 in the oxygenator; this he attributed to a hypoxic vasodilatation. Stehlin (119) and Hottinger (64) also believed the use of 100% O_2 to be undesirable because of the associated rise in pH, and employed a mixture of 95% O_2 and 5% CO_2. Boyland (20) used 97% O_2 and 3% CO_2 and maintained the pH within normal limits (7.3 to 7.4). The consensus of opinion therefore appeared to be that an alkaline pH was undesirable and that a physiologic or acidic pH should be maintained.

TABLE I

Rat No.	pH of Perfusate		Venous line pH
	Oxygenated	Drug Added	
1	7.410	7.373	7.310
2	7.402	7.380	7.345
3	7.425	7.378	7.340
4	7.420	7.320	7.275
5	7.458	7.420	7.310

pH values during standard perfusions. Perfusate samples were taken (1) immediately after perfusate left oxygenator; (2) from the arterial cannula, including admixed drug; and (3) from the venous cannula.

The pH measurements obtained using the standard perfusion indicated that the use of 100% O_2 in the miniature bubble oxygenator did not result in any excessive alkalinity of the perfusate, that which did occur being adequately counteracted by the addition of T.S.P.A. so that the perfusate entering the limb tissues was within physiologic limits.

Leakage.

The problem of perfusate leakage has been alluded to at one time or another by almost every author concerned with the subject of isolation perfusion. Such concern is understandable because the success of the procedure is based on the assumption that a therapeutically significant drug concentration can be maintained in the tumor area while systemic dosage is kept within safe limits. Obviously, uncontrolled and excessive leakage of perfusate would defeat the very aim of the technique. In clinical practice the degree of circulatory isolation of the perfusate has been determined in a number of ways, chief among these being the use of radioactive iodinated serum albumin or RISA (49,66,115, 119-121,139,140), Cr^{51} - tagged red cells (11,28,61,68,89,94), or plasma dyes (94). All these techniques are open to criticism in that they determine circulatory leak, and do not necessarily reflect drug leak. They do however give some indication of the exchange of perfusate between the isolated circuit and the systemic circulation.

Measurement of circulatory leak was performed during

5 consecutive standard hind limb perfusions using Cr^{51}.

Method. 5 rats, each weighing 260 grams, were selected for study to provide uniform body weight. The general technique for perfusion was standard except that an exact quantity of perfusate was used. 8.5 cc of blood was withdrawn from a donor rat into a syringe containing 0.5 cc of Cr^{51} in dilute heparin (approximately 10 microcuries of Cr^{51}). A 1.0 cc sample of this labelled blood was retained as the perfusate sample; the remaining 8.0 cc were placed in the oxygenator. Perfusion was then carried out in the normal way for 16 minutes so that all the perfusate entered the limb. The abdomen of the perfused animal was then opened, and a 1.0 cc sample of blood taken from the abdominal aorta.

In rat C, cannulation of the femoral vein was unsatisfactory. This rat would normally not have been perfused, but was deliberately retained in this study to observe the effect of poor technique on leakage. A good venous return was never established with this preparation, and on two occasions during perfusion the tourniquet was released during attempts to improve flow.

Counts were performed on the samples using a well-type scintillation detector (Model 810-A, Atomic Instrument Co., Cambridge, Mass.) coupled to a suitable rate meter (S.C. 18 Superscaler, Tracerlab Inc., Boston, Mass.). The total rat blood volume was estimated at 5.3 cc/100 grams body weight (41). The blood volume of the isolated limb was estimated at 12.5% of the total blood volume; this estimate was based on

the proportional weight of the limb and was only very approximate.

Results. The counts for each sample and the calculated leakage are shown in Table II. Leakage was calculated according to Austen and Baker (12):-

(1) Since each rat weighed 260 grams, then the total blood volume was $\frac{260 \times 5.3}{100} = 13.8$ cc.

(2) Since the estimated limb volume was 12.5%, then the estimated volume of the systemic circulation was 13.8 less $\frac{12.5 \times 13.8}{100} = 12.1$ cc.

(3) Let S = estimated volume of the systemic circulation, and let P = volume of perfusate.

$$\text{Then } \frac{S \times \text{counts}/3\ \text{min/cc} \times 100}{P \times \text{counts}/3\ \text{min/cc}} = \%\ \text{leakage.}$$

Discussion. The results indicate that good isolation of the perfused limb was achieved with the standard technique, provided that cannulation was satisfactory and a good venous outflow obtained. The sharp increase in leakage resulting from poor venous drainage was in agreement with the findings of others (21). In the good perfusions leakage was remarkably small, the usual leakage with femoral perfusions in humans and dogs approximating 15 to 20% (37). Several factors probably contributed. In the first place the tourniquets used in the rat perfusions were placed deep to the femoral vessels and a very snug fit was obtained. This is not always so in humans

TABLE II

Perfusion	Perfusate		Systemic Circulation		% Leak
	Volume	Counts/3 min/cc	Volume	Counts/3 min/cc	
A	8 cc	207518	12.1 cc	12	0.01
B	8 cc	75526	12.1 cc	0	0.00
C	8 cc	66839	12.1 cc	32775	74.16
D	7 cc	131315	12.1 cc	0	0.00
E	8 cc	80851	12.1 cc	0	0.00

Estimation of circulatory leak using Cr^{51}. All samples counted for 3 minutes; figures quoted are less background reading of 1146 counts/3 min (mean of 8 determinations, range 1116 to 1175 counts/3 min). In perfusion C venous outflow was obstructed.

where the tourniquet has to be placed around the skin of the upper thigh, and held in place by a pin driven into the crest of the ilium. Secondly, the short duration of perfusion (21,121), and the low flow rate with a resultant pressure below systemic values (21,109,121) undoubtedly contributed to minimizing leakage. Thirdly, it may be, as Rochlin has suggested (109), that the pressure differential between the systemic and the perfused circuit allowed the remaining collateral arterial circulation to flow into, rather than out of, the perfused area. If this latter premise was true, the collateral flow must have been very small since there was no obvious quantitative difference between arterial input and venous outflow.

While the number of estimations in this study was small, and the estimates of blood volume only very approximate, so that the experimental error was very large, the finding that in three out of four perfusions the counts/cc of the systemic circulation were no greater than background would seem to justify concluding that leakage was very small.

It was mentioned earlier that estimates of leakage using RISA, Cr^{51} or plasma dyes are open to criticism in that they reflect circulatory leak, which is not necessarily synonymous with drug leak. For example, Shingleton et al., (118) studied leakage during abdominal perfusion in dogs using two indicators: I^{131} - labelled albumin to measure

circulatory leak, and C^{14} - labelled nitrogen mustard to measure drug leak. He showed that leak of the two indicators was significantly different, circulatory leak amounting to 35% while drug leak was only 15%, after 20 minutes of perfusion. He suggested that the extent of drug leak would depend to a great extent upon the speed of reactivity of the specific drug used in perfusion. Buckner et al., (21) used RISA in limb perfusions of rabbits to demonstrate an increased circulatory leak under hyperthermic conditions; this finding is probably valid, although the temperature range was unphysiologic (10 to 50 C) and there was no description in their paper of a tourniquet being used. In contrast, Rochlin et al., (109) using P^{32} - tagged T.S.P.A., showed in dogs that the rate of binding of this drug to the perfused tissues was enhanced by hyperthermia (38 C) so that drug leak would be reduced even in the presence of increased circulatory leak.

It seemed therefore that what was needed was a method for direct assay of chemotherapeutic agents in plasma or blood. Mahaley and Woodhall (93) have recently described techniques for this purpose, and showed that circulatory leak in experimental and clinical perfusions was greater than the amount of alkylating agent detectable in the systemic circulation. They also showed that the amount of isotopically assayed C^{14} - labelled nitrogen mustard in systemic blood samples was consistently greater than the

amount of chemically assayed nitrogen mustard; they suggested that the isotope was not all attached to active drug. While probably the most accurate method of determining leak, drug bioassays are also fallible in that drug in the systemic circulation will be more rapidly metabolized (e.g. by liver), and hence will show a lower concentration than the true leak (28). Other studies by Lawrence and Clarkson (89) using isotopes (Na^{24} and K^{42}) and an agent (Methotrexate) having a predominantly extravascular distribution have further demonstrated that intravascular samples of RISA and Cr^{51} do not accurately reflect the transfer of specific chemotherapeutic agents.

Many of these studies, while interesting academically, involve techniques too complicated for routine use. Stehlin (121,124) has emphasized that the need is for a simple technique permitting continued monitoring of leakage during perfusion, so that adjustments in dose, time and isolation may be made according to the leakage demonstrated. Because it is simple to handle and requires little time for preparation, Stehlin has continued to use RISA and comments that: "Despite obvious sources of error, RISA studies have almost uniformly afforded excellent correlation between the leakage factor and the anticipated degree of bone-marrow depression postoperatively."

Definition of Standard Perfusion

As a result of the data and theory described above,

the standard perfusion of the rat hind limb bearing the Walker 256 carcinoma was defined as one in which:

(a) the perfusate was fresh heparinized rat blood having an oxygen saturation of not less than 95%, maintained at 37.0°C in the arterial cannula and having a physiologic pH.

(b) perfusion was continued for 15 minutes at a flow rate of 0.5 cc/min thus maintaining an arterial line pressure below systemic pressure with negligible leak of perfusate into the systemic circulation.

(c) 4 day old tumors were perfused with T.S.P.A., 4 mgm/kg body weight.

PART 2. METHOD OF STUDYING TUMOR RESPONSE TO PERFUSION.

In the studies reported in the remainder of this thesis the effect of perfusion was decided on the basis of: (1) alteration of animal survival time; (2) the response of the primary tumor; (3) change, if any, in the metastatic pattern observed at autopsy, and (4) evidence, if any, of local toxicity within the perfused limb.

Animal Survival Time.

All survival times were calculated in days from the day of tumor inoculation, not from the day of treatment.

Tumor Size.

Measurements of tumor size were made using a metal caliper. Only the greatest measurable diameter of the tumor mass was recorded; because of the situation of the

tumor this necessarily included overlying skin and muscle. Serial measurements of tumor size were necessary because death of the animal was associated with a variable diminution in size resulting from ulceration of, and hemorrhage from, the tumor; thus the tumor size at autopsy bore no consistent relation to the maximum size attained during life.

Metastatic Pattern.

Metastases from the Walker tumor as used in this experiment were invariably limited to the abdominal para-aortic nodes and to the lungs. Metastases were never seen in the liver or other abdominal viscera, although occasionally a kidney or adrenal gland would be involved in a confluent mass of nodes.

The incidence and extent of metastases were recorded in all animals at autopsy. Because of the large numbers of animals involved, routine microscopic studies were not done; a separate study of the histopathologic features of primary and metastatic tumor is reported in Chapter 5. When metastases were present on gross examination, the number and size were recorded.

Pulmonary metastases were graded according to the total number of surface metastases visible with the naked eye. The lungs were removed intact, washed, and examined under a good light. An arbitrary grading of 0 to 4 + was used:

0 : no metastases visible.
1 + : less than 10 visible surface tumors.
2 + : less than 30 visible surface tumors, but more than 9.

3+ : less than 50 visible surface tumors, but more than 29.

4+ : over 50 visible surface tumors.

These grades are illustrated in Fig. 8. The examples of 2+ and 3+ lungs were deliberately chosen to demonstrate that a superficial glance at one aspect of the lungs could be misleading.

Nodal metastases were graded according to the diameter of the nodes. In all cases the nodes were dissected out and the two greatest diameters recorded. Because of variability in shape the following empirical grading was used:

0 : apparently uninvolved node; mean diameter less than 0.75 cm., no diameter greater than 1.0 cm.

1+ : mean diameter less than 1.0 cm; no diameter greater than 2.0 cm.

2+ : mean diameter greater than 1.0 cm but less than 2.0 cm; no diameter greater than 2.0 cm.

3+ : any diameter greater than 2.0 cm; no diameter greater than 3.0 cm.

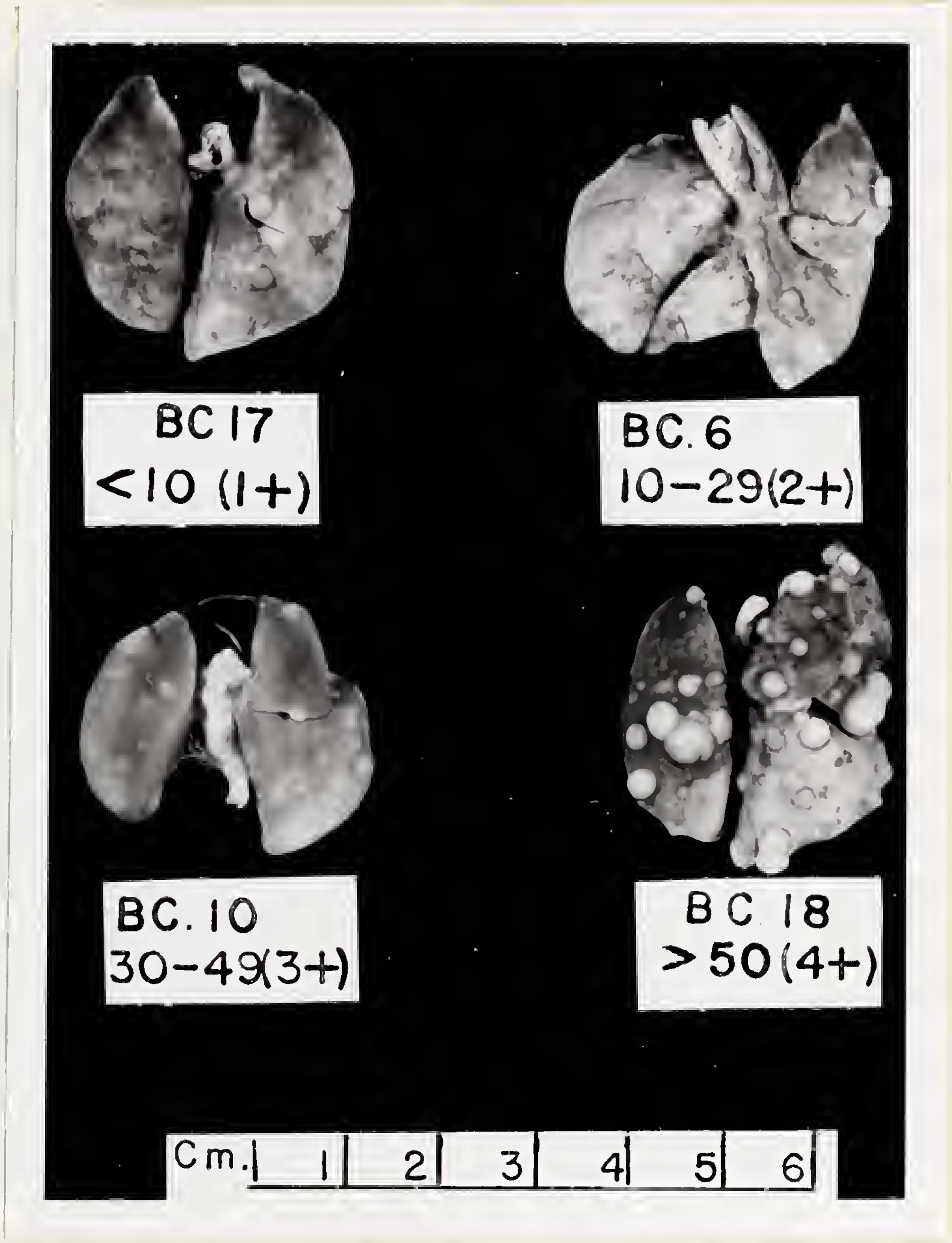
4+ : any diameter greater than 3.0 cm.

These grades are illustrated in Fig. 9.

Toxicity within the Perfused Limb.

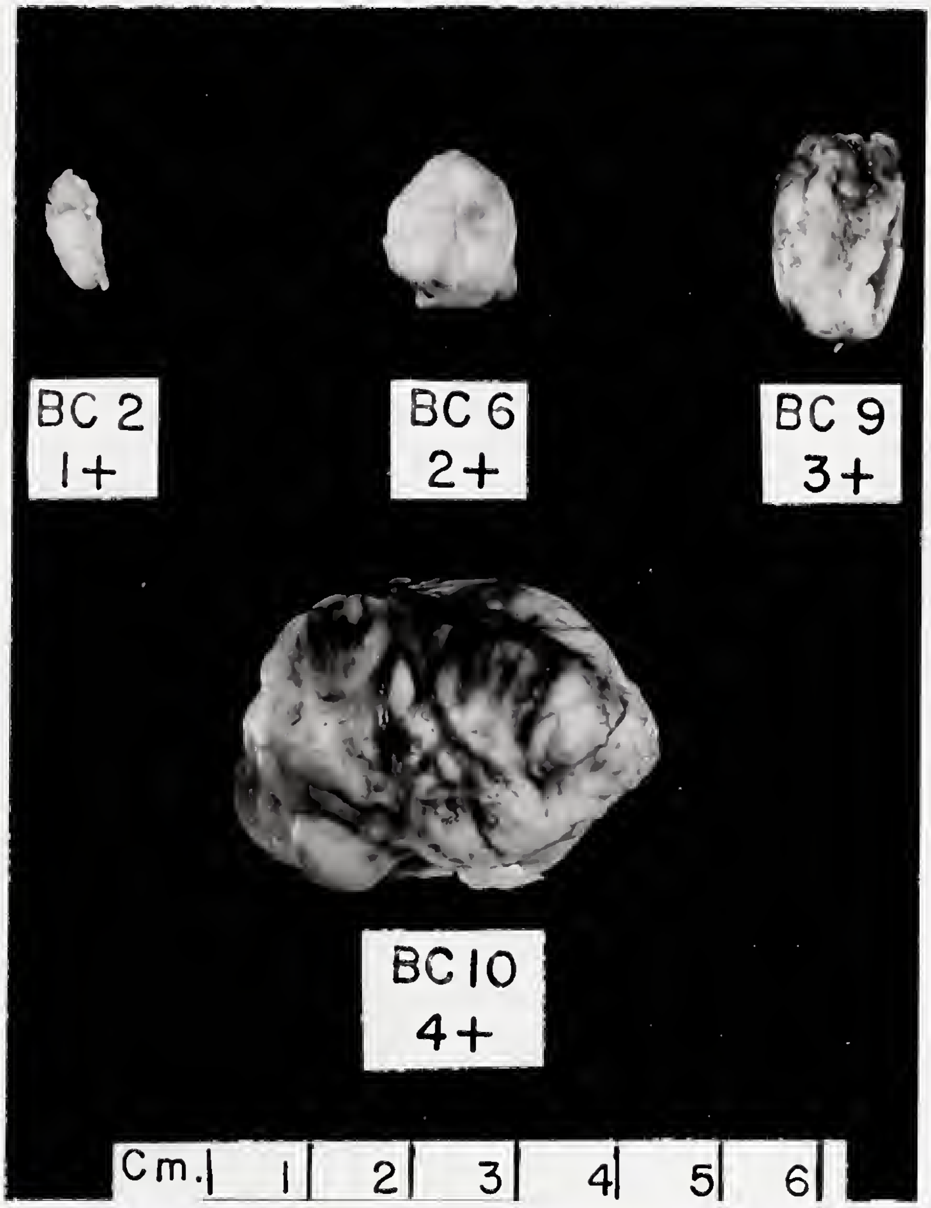
The fundamental objective of any form of cancer chemotherapy is selective destruction of malignant tissue. It was therefore important that, in assessing the effect of perfusion, a careful evaluation be made of damage to normal

FIGURE 8



Examples of the different grades of lung metastases. Note that the difference between 1+, 2+ and 3+ is not readily apparent when only one surface of the lungs is viewed.

FIGURE 9



Examples of the different grades of nodal metastases. For the criteria used in grading, see text.

limb tissues. Accordingly the condition and function of all limbs were assessed on the third day after surgery, and at regular intervals thereafter. Following perfusion there was usually a transient generalized edema of the limb lasting 24 to 48 hours, during which period there was some degree of spontaneous limitation of limb movement. Thereafter, in the absence of local tissue damage, limb function was indistinguishable on gross examination from that of untreated controls.

Damage was considered to have occurred if:

(1) there was any loss of normal limb function;

(2) there was any macroscopic evidence of paralysis, muscle wasting or soft tissue necrosis.

The commonest form of damage was necrosis of the terminal parts of the digits.

PART 3. THE EFFECT OF THE STANDARD PERFUSION ON THE WALKER TUMOR.

Having defined the standard perfusion and the method of evaluating its effectiveness, the typical response of the Walker tumor to perfusion with and without T.S.P.A. will be described.

Method.

Male Sprague-Dawley rats weighing 250 to 350 grams were used. The Walker tumor was transplanted in the usual manner. 150 rats were studied, these being divided into 3 groups of 50 rats each:

(1) Group A rats were perfused with T.S.P.A., 4 mgm/kg under

the conditions of the standard perfusion.

(2) Group B rats were perfused in an identical manner without any drug.

(3) Group C rats remained untreated as controls.

All animals were kept under identical conditions, observed regularly, and careful records made of survival time, tumor size, metastatic pattern and limb condition. No further treatment was given to any animal.

Results.

The results are presented in Table III.

Animal survival time. The differences in survival time between the three groups were in all cases significant at the $P < 0.05$ level. However, perfusion with T.S.P.A. had a much more profound effect than perfusion in the absence of drug.

There were 2 spontaneous regressions in group A, 1 in group B, and 2 in group C. These animals were not included in calculations of survival time. The question of spontaneous regressions is discussed in Chapter IV and will not be pursued here. The remaining discrepancy in numbers was due to anesthetic deaths.

Tumor size. Serial measurements of tumor size demonstrated that perfusion with T.S.P.A. resulted in apparent cessation of growth of tumor for a variable period of 7 to 17 days (Fig. 10). Thereafter growth resumed and followed the characteristic pattern seen in untreated animals. This temporary discontinuance of growth was not seen in animals perfused without

TABLE III

Group	No. of rats Evaluated	Survival (days)		Incidence	Lung Metastases		Node Involvement	
		Mean	Range	Limb Damage	Incidence	Ave. Grade	Incidence	Ave. Grade
A	47	39	32-52	2%	91%	2.0	65.0%	2.0
B	49	24	17-29	2%	100%	2.5	87.0%	2.3
C	48	21	18-26	0%	98%	2.4	82.0%	2.4

Effect of perfusion with and without T.S.P.A. on the Walker tumor.

Group A: perfused with T.S.P.A., 4 mgm/kg.

Group B: perfused without T.S.P.A.

Group C: untreated controls.

T.S.P.A.

Limb damage. The incidence of limb damage was negligible in all groups; in both perfused groups, 1 rat had minimal ischemic necrosis of the tips of two digits.

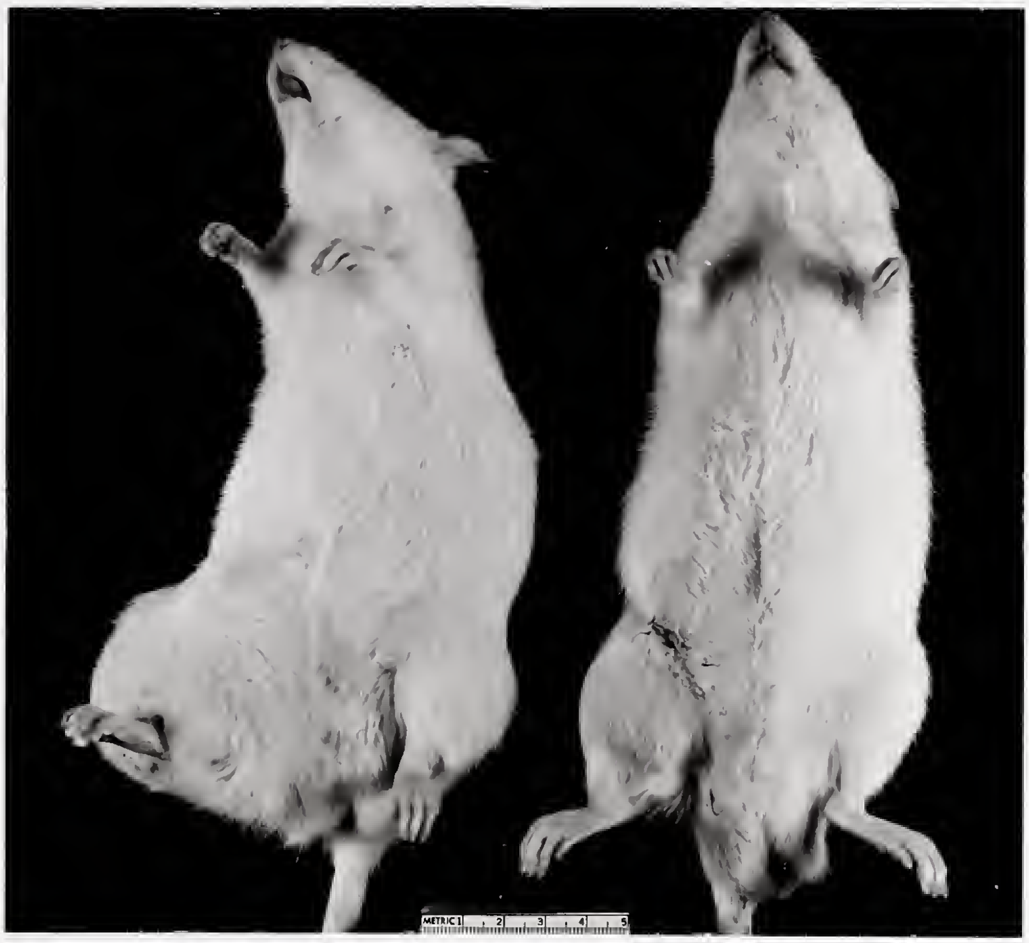
Metastases. The metastatic patterns were essentially similar in all groups, although the incidence of both pulmonary and nodal metastases was least in the animals perfused with drug. Perfusion without drug did not materially alter the pattern from that seen in untreated controls.

Discussion.

These results showed that perfusion without any chemotherapeutic agent would slightly, but significantly, prolong survival time. Presumably this was the result of a degree of temporary derangement of tumor metabolism, suggesting that the standard perfusion was not quite as physiologic a procedure as was hoped. Ligation of the main vessels of supply could not be inculpated, since this has been frequently shown to have no effect on otherwise untreated controls (53).

Perfusion with T.S.P.A. did reduce the incidence of metastases. This result was probably more significant than the figures suggest, because these rats lived an average of 15 days longer than controls. Thus the opportunity for the establishment and growth of metastases was considerably greater. The reduced incidence of metastases must be

FIGURE 10.



Comparison of tumor size 20 days after perfusion with T.S.P.A., with that of untreated control. The treated tumor (right) is just beginning to resume growth.

attributed to the effect of T.S.P.A., since in all other respects conditions of the experiment were identical for groups A and B. While at one time the reduced incidence of metastases, when comparing groups A and C, was thought to be an effect of heparin which has been shown to reduce the "take" of circulating cancer cells (2,29,42), this factor could not apply in a comparison of groups A and B. Since leakage was shown earlier to be negligible under the conditions of the standard perfusion, then the reduction in metastases must have resulted from the effect of T.S.P.A. on the primary. Presumably few or no viable cells were disseminated during the period of cessation of tumor growth.

Summary.

Standard perfusion of the Walker tumor approximately doubled animal survival time, and reduced the incidence of both pulmonary and nodal metastases. Perfusion without T.S.P.A. also significantly prolonged life in a statistical sense, but to a much lesser degree. Limb damage was negligible.

PART 4. STUDIES ON TEMPERATURE AND OXYGEN SATURATION IN ISOLATION PERFUSION CHEMOTHERAPY.

In this section two studies are presented in which the technique of isolation perfusion was used to evaluate the effect of alteration of tumor environment on the efficacy of the antitumor activity of an alkylating agent.

(1) An Experimental Study of Temperature in Isolation Perfusion Chemotherapy.

One of the more easily controlled variables in isolation perfusion is the temperature of the perfusate. Woodhall et al., (21) suggested that since the complex processes of a highly organized living cell would conform to the kinetics of a single reaction such as temperature, then the effect of any antitumor substance upon a tumor cell should be increased in the presence of a hyperthermic environment. Confirmatory studies using the VX 2 carcinoma (92) led Woodhall to employ perfusate temperatures of 42°C in perfusing cancers of the head and face (140). Rochlin et al., (109) using P^{32} - tagged T.S.P.A. demonstrated a direct correlation between temperature and the rate of binding of the chemotherapeutic agent to the perfused tissue, high local tissue temperature (38°C) markedly increasing the amount of drug bound. Ausman and Aust (9) found that one-third of the total activity of nitrogen mustard was lost within the first five minutes of exposure to blood at 37°C., whereas only 10% of the activity was destroyed in a similar period at 7°C. On this basis it was hoped that hypothermia might be of some advantage in increasing the total amount of nitrogen mustard absorbed by any given area, but it was found that hypothermia reduced the metabolic action of the tissue sufficiently to impair the process of absorption and inactivation of mustard in the hydrolyzed form, so that the

net result of perfusion at hypothermic temperatures was no improvement over perfusion at physiologic body temperature.

Shingleton and others (118,141) combined a hyperthermic perfusate to enhance drug reaction with hypothermia of tissues outside the perfused area to protect the bone-marrow, in perfusions of abdominal and intra-thoracic neoplasms where only poor vascular isolation could be achieved and a correspondingly large leak of anticancer agent might be expected. Shingleton has also demonstrated the protective effect of hypothermia against lethal doses of mechlorethamine in dogs (117).

The consensus of opinion, therefore, was that hyperthermic perfusate temperatures were preferable in view of theoretical and in vitro evidence of enhanced activity of anticancer agents under hyperthermic conditions. There was, however, no direct in vivo experimental evidence to support this hypothesis, and it was decided to investigate the effect of alteration in perfusate temperature on the response of the Walker carcinoma to perfusion with T.S.P.A. In addition, studies were also undertaken to evaluate the use of externally applied heat in the form of local diathermy as had been advocated by Young (141).

Methods.

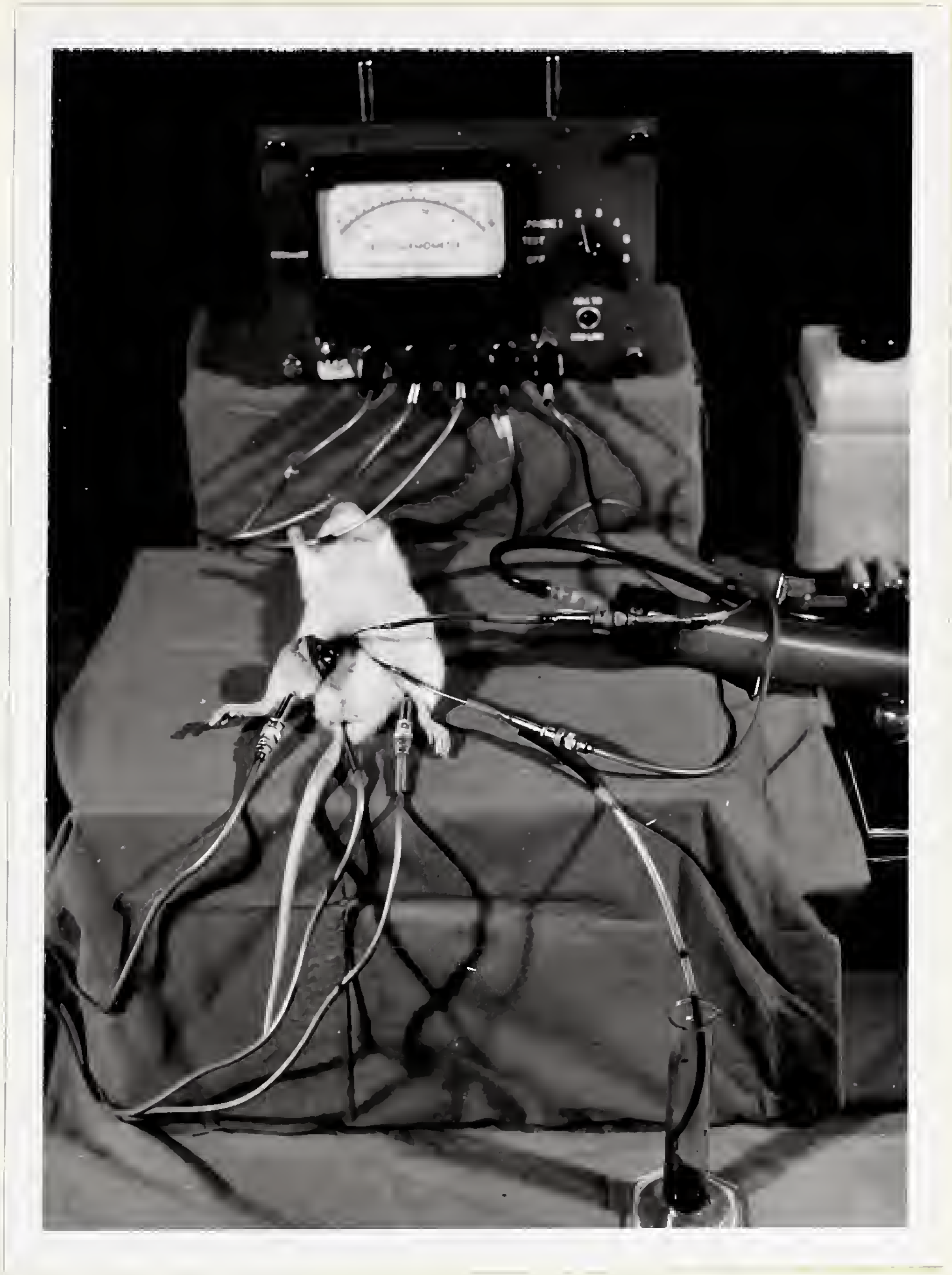
Part 1. Six groups of 25 rats each were studied. Groups 1, 3 and 5 were perfused with T.S.P.A., 4 mgm/kg under standard conditions but with perfusate temperatures of 42°, 37° and 22°C respectively. Groups 2, 4 and 6 were perfused without drug

at 42°, 37° and 22°C respectively. A further group of 50 rats (group 7) remained untreated to act as controls. All rats were kept in individual cages and fed a standard laboratory diet with water ad libitum. Survival time, gross tumor response, metastatic spread and limb viability were studied in the manner described previously.

Part 2. Six groups of 3 rats each were perfused under the same conditions as groups 1 to 6 in Part 1. In these animals a study was made of the temperature changes within the tumor and the perfused limb during perfusion. Thermistor probes were placed in the arterial and venous lines, in the rectum, in the tumor and subcutaneous tissues of the tumor limb, and in the posterior muscle mass of the contralateral hind limb (Fig. 11). Temperatures were recorded at 2 minute intervals over the 15 minute period of perfusion, and in one animal from each group perfusion was continued for 30 minutes while temperatures were recorded. In a further 3 rats the temperature within the tumor was monitored during the procedure of femoral vessel cannulation and application of the tourniquet These animals were killed after the pertinent observations were completed.

Part 3. 30 rats were perfused with T.S.P.A. while the tumor was being heated with externally applied diathermy; the perfusate temperature was maintained at 37°C in this series. These rats were maintained and observed in a similar manner to those in Part 1 of this study. In a further 6 rats

FIGURE 11.



Photograph to illustrate position of thermistors. These were placed in the rectum, in the tumor, in the posterior muscle mass of the contralateral hind limb, and in the arterial and venous cannulae. A further probe, not illustrated here, was placed in the subcutaneous tissues of the perfused limb.

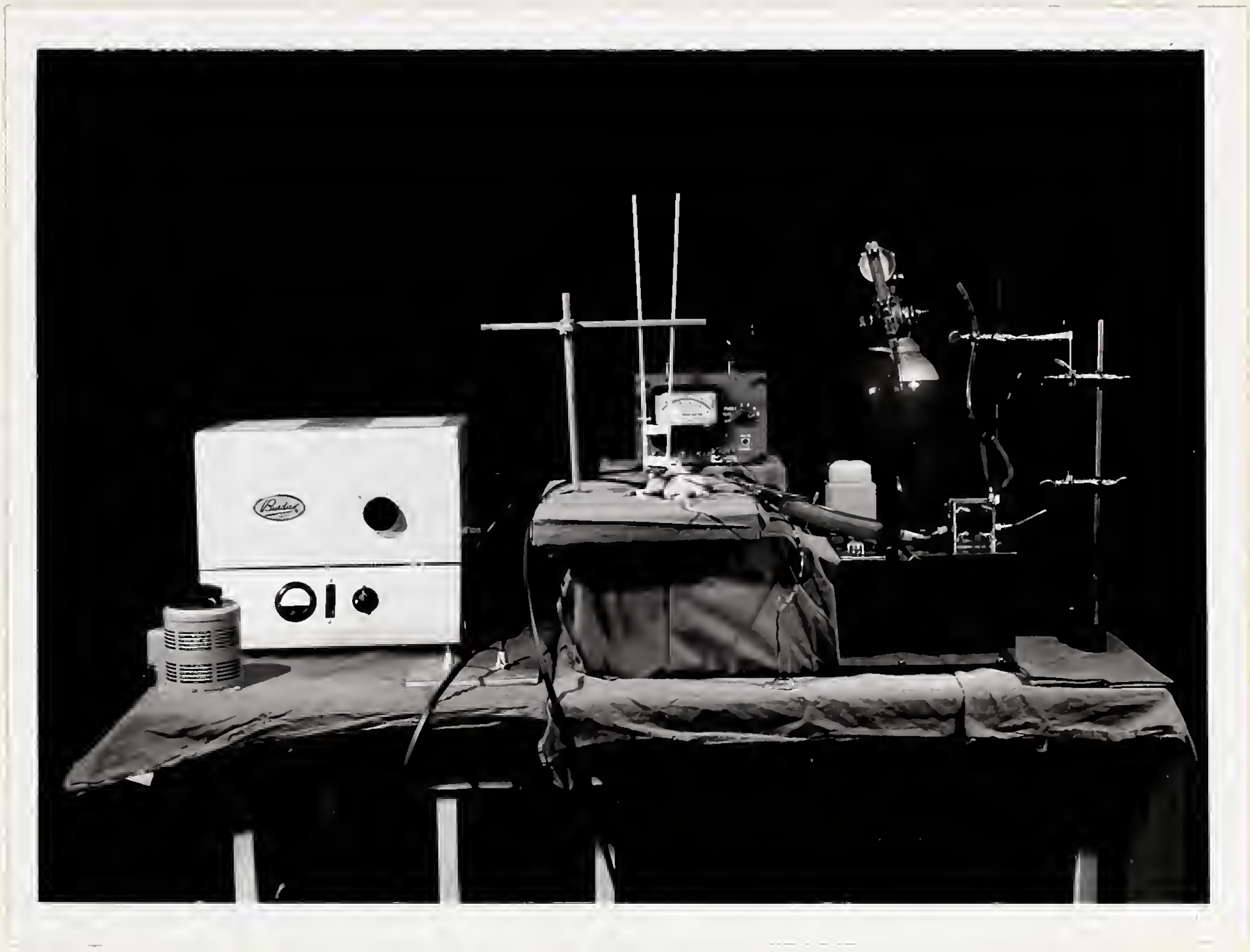
temperature measurements were made within the limb during perfusion; the diathermy was temporarily discontinued while these measurements were made, since excessive and inaccurate heating of the metal probes would otherwise have occurred.

The diathermy machine was a modified Burdick Model D-54 (The Burdick Corporation, Milton, Wis.). In the standard model the plate and filament voltages were supplied by secondary coils which had a common primary coil, and hence any change in applied voltage to the primary coil resulted in voltage changes in both secondary coils. Since this would cause undesirable changes in the temperature of the filament, the machine was modified by installing a separate transformer to supply filament power, its primary being connected directly across the power line. Voltage supplied to the plate of the tube was controlled by a variable ratio transformer, providing very smooth control of R.F. output. R.F. output was supplied to the load using 300 ohm twin line lead (Fig. 12).

Results.

Part 1. The survival time in days and the incidence of limb damage in the various groups are shown in Table IV. An unusually high incidence of anesthetic deaths accounted for the reduction in numbers in each group. In each of groups 1, 5 and 7 one rat survived over 100 days; these rats were sacrificed and no evidence of tumor found. These were

FIGURE 12.



Diathermy machine in use during perfusion. The diathermy machine and variable ratio transformer are to the left of the picture. The heating pads are in position above and beneath the perfused tumor.

considered to represent spontaneous regressions and were not included in calculations of survival time etc., (see also Chapter IV).

The survival time of all rats treated with T.S.P.A., irrespective of perfusate temperature, was significantly increased over that of controls ($p < 0.01$). However the small differences in survival time between the groups treated with T.S.P.A. at 42°, 37° and 22°C were not statistically significant. Limb damage was slight and in no case amounted to more than necrosis of the terminal parts of one or two digits. Limb function was well preserved. No significant differences were observed in the incidence or extent of metastases between the various groups.

Part 2. The results of this study were of some interest. It was found that the tumor temperature dropped as much as 3°C over the 5 minute period taken to expose the femoral vessels, ligate the artery and insert the cannula. A further drop of 1°C followed application and tightening of the tourniquet. Room temperature during these procedures was 22°C. Thus the usual tumor temperature before perfusion was in the region of 32° to 33°C. Subsequent alteration in tumor temperature with perfusion was not as great as had been expected. Perfusion for 15 minutes with a perfusate temperature of 42°C only succeeded in raising the tumor temperature to 34°C. Perfusion at 37°C resulted in a further slight drop in tumor temperature to about 32°C. With a perfusate temperature of 22°C the tumor temperature dropped to about 27°C but was never as cold as the perfusate. Examples of the various recorded temperatures

TABLE IV

Group	No. of Rats	Perfusate Temperature	T.S.P.A.	Survival (days) Mean	Range	Incidence Limb Damage.
1	24	42.0 C	+	39*	28-49	9%
2	23	42.0 C	-	24	20-27	4%
3	23	37.0 C	+	38	31-52	4%
4.	24	37.0 C	-	24	20-29	0%
5	24	22.0 C	+	37*	30-45	4%
6	23	22.0 C	-	24	17-31	4%
7	50	Untreated Controls		20*	17-25	0%

*One long term survivor in this group (greater than 100 days) not included in calculation of survival time.

Comparison of survival times and incidence of limb damage in groups of rats with Walker tumor perfused with and without T.S.P.A. (4 mgm/kg) at 42°, 37° and 22°C.

at the conclusion of a 15 minute perfusion are shown in Table V. These figures were not greatly altered when the perfusion was prolonged to 30 minutes. The presence or absence of drug did not affect the temperature changes.

Since the subcutaneous temperature of the limb did respond to perfusate temperature, it was concluded that much of the perfusate must bypass the tumor vascular bed. To substantiate this observation, arteriograms were made using a solution of 50% sodium diatrizoate heated to 42°C. These showed that relatively little dye entered the tumor vascular bed (Fig. 13).

Part 3. 2 rats in this experiment succumbed to the anesthetic. 1 rat was a long term survivor. The other 27 rats had a mean survival time of 35 days. The incidence of limb damage was greatly increased, occurring in 70% of the rats treated. This took the form of superficial soft tissue burns (Fig. 14), and/or neuromuscular damage resulting in flaccid paralysed limbs. Observations of temperatures within the limb showed that it was necessary to heat superficial tissues to 48°C in order to achieve a tumor temperature of 42°C., a temperature gradient being created through the limb. Survival time and limb damage in the diathermy group are compared with those for rats perfused at 42°C and untreated controls in Table VI.

Discussion.

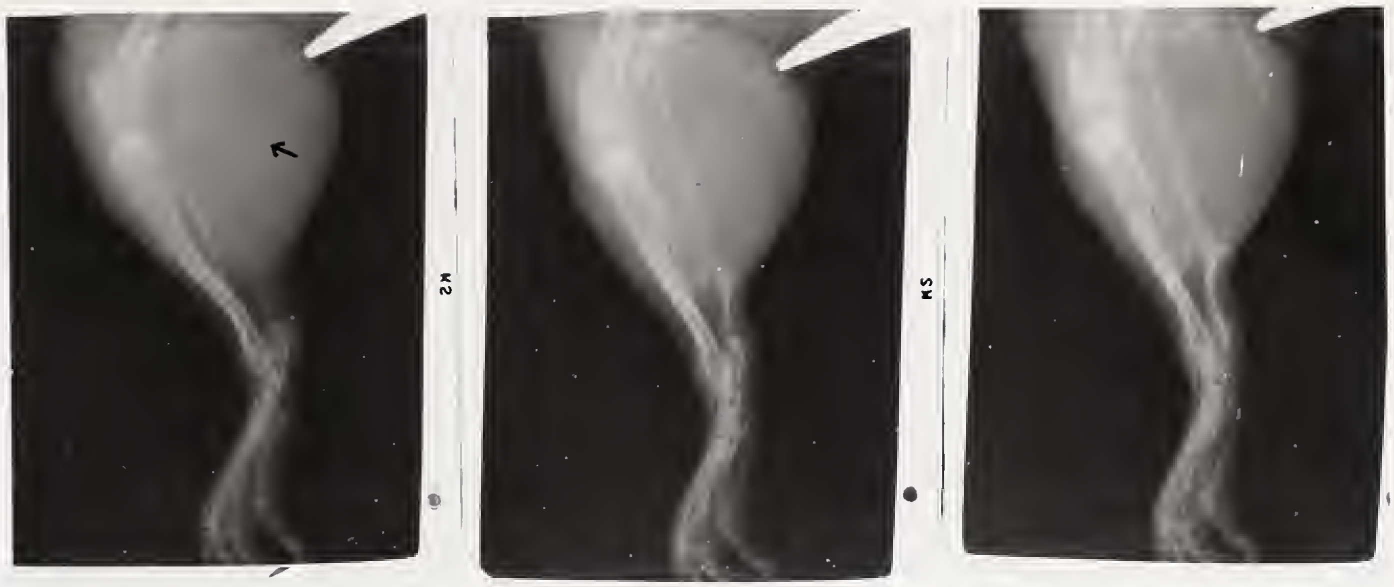
The conclusions that could be drawn from this study were limited but important. It was apparent that

TABLE V

Perfusate	Tumor	Venous Outflow	Subcutaneous	Contralateral Limb	Rectum.
41.8	34.4	36.0	40.0	36.7	37.2
37.2	32.6	32.8	36.2	36.4	37.2
22.0	26.3	27.7	28.5	35.0	35.4

Temperatures recorded at the conclusion of a 15 minute perfusion (°C).

FIGURE 13

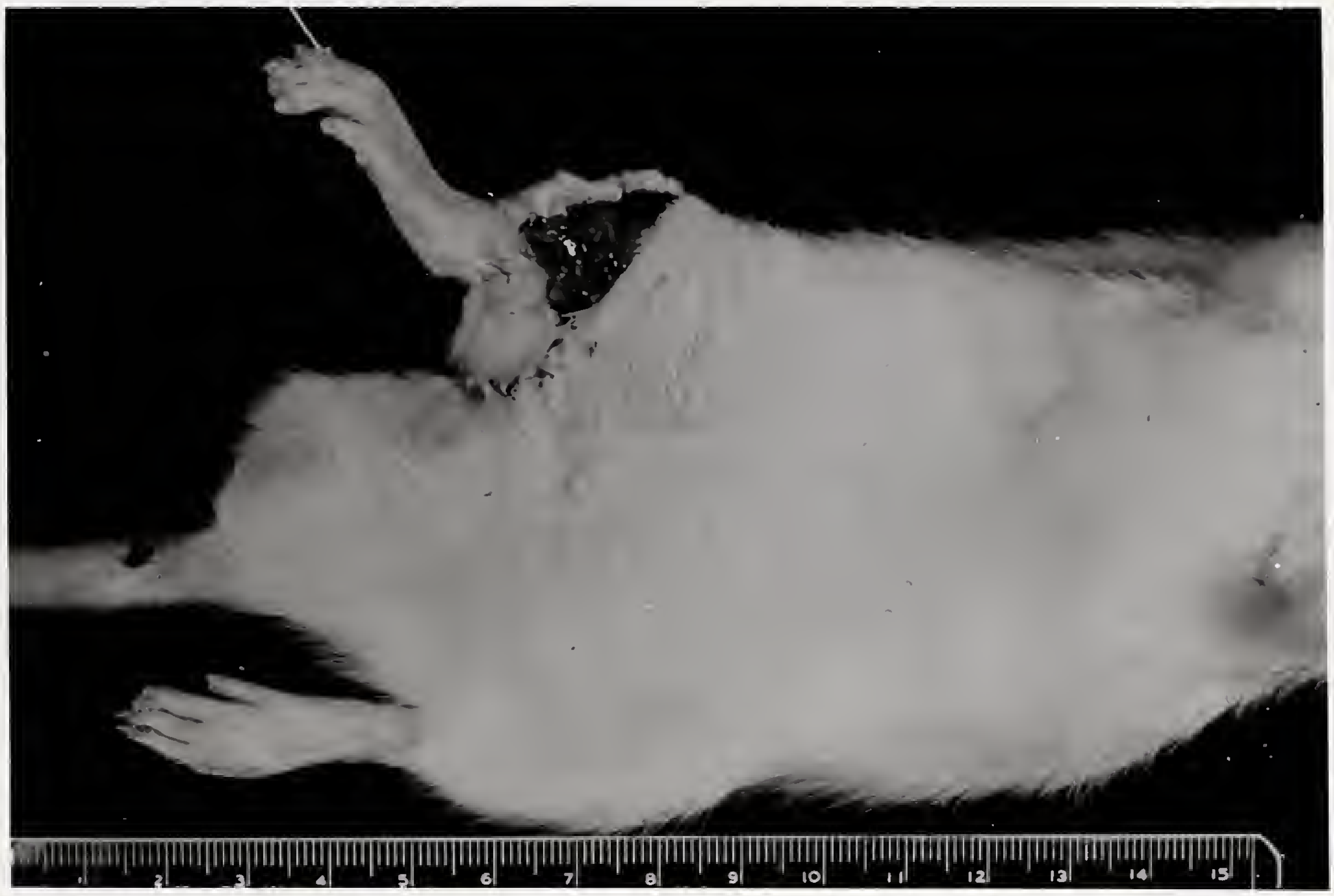


Arteriograms of isolated limb made using 50% sodium diatrizoate heated to 42°C. Arrow indicates position of tumor. Exposures made at 1 sec intervals reading from left to right. Note that very little dye entered the tumor vascular bed.

perfusate temperature and tumor temperature were not necessarily synonymous in the system studied. Because the subcutaneous tissues of the limb responded to perfusate temperature, while the tumor did not, it was postulated that much of the perfusate might bypass the tumor vascular bed. In other words, histological evidence of vascularity did not neccessarily bear any relation to blood flow through the tumor. An attempt was made to confirm this by radiographic means, the resulting arteriograms suggesting that little of the perfusate actually traversed the tumor vascular bed. Recently, ingenious studies by Gullino and Grantham (54), have furnished evidence that the blood flow through a variety of small animal tumors, including the Walker tumor grown in rat kidney, is some fifteen to twenty-fold less than that of the host organ. These results confirmed the author's impression, and suggest that chemotherapy must be re-evaluated in terms of tumor blood flow. Certainly they help to explain why tumor temperature varied only 8°C, despite a perfusate temperature range of 20°C, in the present study. It is not surprising, therefore, that the tumor response, as measured in terms of animal survival time, was not significantly different in the several groups.

The second conclusion drawn from this study was that use of externally applied diathermy to heat deep-seated tumors was of no practical value. The use of diathermy was explored after it became apparent that tumor temperature was

FIGURE 14



Typical soft tissue burn 7 days after perfusion with T.S.P.A. while tumor was being heated with diathermy.

TABLE VI.

Group	No. of Rats	Survival (days) Mean	Survival (days) Range	Incidence Limb Damage
Diathermy	27	35	26-46	70%
Perfusate 42 C	24	39	28-49	9%
Controls	50	20	17-25	0%

Comparison of survival times and incidence of limb damage in rats perfused with T.S.P.A. using (1) external (diathermy) heating, and (2) perfusate heated to 42°C.

not being materially altered by varying perfusate temperature. Young (141) had previously suggested that the use of local diathermy to maintain hyperthermic conditions in the region of the tumor should be evaluated. Unfortunately diathermy, as used in this experiment, would appear to do more harm than good. In order to heat deeper tissues to the required 42°C, it was necessary to heat intervening superficial tissues as high as 48°C. This resulted in extensive local burns and evidence of neuromuscular damage to the limb, while the tumor response was not enhanced in any way. This bears out the conclusion reached by Gilchrist and his co-workers (46,96) that diathermy was theoretically unsuitable for producing localized heating in intact animals. To achieve this object Gilchrist prefers to use a target material in the form of minute particles of magnetic iron oxide, which undergo heating as a result of hysteresis loss when exposed to a suitable radiofrequency. This technique works well when attacking lymph nodes which will concentrate the injected iron oxide particles, but a more general application would seem difficult.

The more general question of whether or not hyperthermic conditions enhance the activity of anticancer agents in vivo remains unanswered. The difficulty of maintaining a histologically vascular tumor at even normothermic temperatures was apparent, and since theoretical and in vitro

evidence points toward the value of hyperthermic conditions, it would seem advisable to maintain at least a normothermic perfusate.

(2) The Effect of High and Low Perfusate Oxygen Saturation on Tumor Response to T.S.P.A.

When Creech and his associates introduced their technique for regional perfusion (30,31,110), they incorporated a bubble oxygenator into the perfusion circuit because it was felt that oxygenation of the blood would help to sustain tissues of the isolated area, and because the development of a high oxygen tension in the perfusate might potentiate the action of alkylating agents. In these early perfusions, the duration of perfusion was short, isolation was incomplete and hypothermic perfusates were used, so that little injury to normal tissues would have occurred even had unoxygenated blood been used. Krementz and Knudson (87) however, have emphasized that present day perfusions are carried out under hyperthermic conditions, with more complete isolation, for longer periods and with highly reactive agents, so that the use of an oxygenated perfusate is probably necessary to minimize tissue damage.

More controversial is the effect of an increased tissue oxygen tension (pO_2) on the action of alkylating agents. In 1946 Gilman and Philips (47) described the biological actions of alkylating agents as being radiomimetic,

and more recently Alexander and Stacey (3) have suggested that both alkylating agents and ionizing radiation act by breaking or cross-linking the polynucleotide chains of DNA, thereby rendering the DNA molecules useless to the cell. The main differences in biological action between radiation and the alkylating agents follow from the fact that the latter must diffuse into the cell, and that some DNA molecules are therefore more accessible than others to the reagent. The experimental work of Gray and Scott (51,52) and the clinical investigations of Churchill-Davidson and his colleagues (27) provided good evidence that the effect of irradiation on normal and malignant tissues was potentiated by an increase in tissue pO_2. In view of the described similarity of action, it was thought that what applied to radiation might also hold true for the alkylating agents, and that use of a bubble oxygenator in the perfusion circuit would raise tissue pO_2 sufficiently to potentiate the action of alkylating agents.

Churchill-Davidson (27) explained the potentiating effect of an increased pO_2 on the following grounds. In the first place the evidence of Gray et al., (51) has shown that regions of poor oxygenation within neoplasms render some cells so insensitive to radiation damage as to survive the usual forms of radiotherapy and cause later recurrence of growth. Secondly, oxygen reaches the cells of tumors by way of the blood vessels of the stroma and in many, though not all, tumors the cells form masses or cords

surrounded but not penetrated by capillaries. Since oxygen reaches these cells by diffusion, its concentration around any particular cell will depend on each of four factors:

(1) The co-efficient of diffusion of oxygen in tumor tissue, which probably is little different from that of muscle or fibrous tissue.
(2) The oxygen tension in that part of the capillary which constitutes the principal source of oxygen to the cell in question.
(3) The distance of the cell from the capillary.
(4) The respiratory rate of the intervening tumor cells.

Of these only the second lends itself to controlled alteration. Churchill-Davidson attempted such alteration by increasing the partial pressure of the oxygen respired, having his patients breath oxygen at 3 atmospheres.

When air is breathed at sea level the arterial pO_2 is approximately 100 mmHg; there is little loss of oxygen from the arteries so that blood reaching the capillaries also has a pO_2 of 100 mmHg. When pure oxygen is breathed at 3 atmospheres the arterial pO_2 increases to about 2000 mmHg., but the percentage increase in the amount of oxygen carried is comparatively small, almost all the additional oxygen being carried in simple solution in the plasma. When blood under high pO_2 reaches the capillaries, the additional oxygen rapidly diffuses out and the pO_2 falls very quickly to a level in equilibrium with saturated hemoglobin. Beyond this point the fall is again governed by the dissociation curve of hemoglobin, as it is in blood under normal pO_2.

The net result of increasing pO_2 is, therefore, that hemoglobin begins to liberate oxygen at a point some way from the arterial end of the capillary, instead of at the arterial end, as would occur with normal pO_2. Churchill-Davidson admits, however, that since slowing of capillary blood flow is a feature of many tumors, this point of beginning hemoglobin oxygen liberation will not be very far from the arterial end of the capillary.

Gray (52) pointed out that the increase in sensitivity (of Ehrlich ascites cells) to radiation, for a given increment of pO_2 is greatest over the range of pO_2 from 0 to about 100 mmHg. Thereafter a large increase in pO_2 is needed to increase sensitivity very slightly. It has been shown that a bubble oxygenator with 100% oxygen will increase pO_2 of the perfusate to 400-600 mmHg (86). Thus, considerably less oxygen will be in simple solution than that resulting from breathing oxygen at 3 atmospheres, and the point at which hemoglobin begins to liberate oxygen will be correspondingly closer to the arterial end of the capillary. It would seem unlikely, therefore, that the increase in pO_2 achieved with a bubble oxygenator would be of any real significance in enhancing tumor response to alkylating agents. On the other hand the report by Hultborn and Forssberg (65) suggested that simple inhalation of oxygen, a manoeuvre which will increase arterial pO_2 to levels similar to those obtained with a bubble oxygenator, was of some value during radiation of skin tumors.

It seemed worthwhile, therefore, to study the effect of widely different oxygen tensions on the response of the Walker tumor to perfusion with T.S.P.A. This was accomplished by using blood with an oxygen saturation of 10 to 15% and a correspondingly low pO_2, and blood with an oxygen saturation of 100% which had been through a bubble oxygenator and had a high pO_2. While no instruments were available for measuring pO_2, it is reasonable to assume that the difference in pO_2 between the two perfusates was in the order of 400 to 500 mmHg, and included the range, described by Gray, over which greatest increase in sensitivity occurred.

Method.

60 rats with Walker tumor were studied. These were divided into 3 groups:

(1) Group A. 20 rats were perfused under standard conditions, using blood that had been through the bubble oxygenator.

(2) Group B. 20 rats were perfused under standard conditions using oxygen desaturated blood.

(3) Group C. 20 rats remained untreated as controls.

The blood used for group B perfusions was obtained from the right ventricle of donor rats (see Chapter II). It was then placed in a reservoir under a stream of 100% nitrogen, from which it was pumped directly into the perfusion circuit. Oxygen saturations were determined by the method of Van Slyke (136).

After perfusion the rats were maintained and observed in the usual manner until death.

Results.

Oxygen saturation. Measurements of oxygen saturation of the perfusate in group A perfusions indicated that the blood was consistently 100% saturated. The additional oxygen in solution was not measured. The oxygen saturation of the perfusate in group B perfusions had a mean value of 13.6% with a range of 11 to 15%.

Survival time. A comparison of the survival times of the three groups is shown in Table VII. Use of 100% oxygen saturated blood significantly increased survival time ($P < 0.02$).

Limb Damage. Both perfused groups included one rat with partial loss of function in the perfused limb. There was no evidence of any ischemic changes in the limbs perfused with O_2 desaturated blood.

Metastases and tumor size. The metastatic patterns observed at autopsy were similar in the two treated groups, and bore the same relation to metastases in untreated controls as was described earlier. Tumor size was similar in the treated groups at death, but the tumors perfused with O_2 desaturated blood only regressed for 6 to 12 days compared with the 7 to 17 day regressions seen in tumors perfused with 100% O_2 saturated blood.

Discussion.

The rationale for oxygenation of the perfusate, as originally expressed by Creech et al., (30) has been explained. In support of their hypothesis, they succeeded

TABLE VII

Groups	Perfusate O_2 Saturation.	No. of Rats.	Survival (days)		Incidence of Limb Damage	Metastases.
			Mean	Range		
A	100 %	20	39	32-52	5%	Unchanged
B	13.6%	20	37	31-43	5%	"
C	Untreated.	20	21	17-27	0	"

Comparison of results obtained after perfusion with T.S.P.A. (4 mgm/kg) using (1) 100% O_2 saturated blood, and (2) 13.6% O_2 saturated blood as perfusate. The difference in mean survival time between groups A and B is significant ($P < 0.02$).

in showing during perfusions of the hind limbs of dogs that increasing tissue pO_2 by 35% increased the toxicity of 5-Fluorouracil by 20% (112). Krementz et al., (86,87) have demonstrated that the cancericidal effect of nitrogen mustard on an Ehrlich ascites tumor in mice was potentiated by increased tissue pO_2, although they do not report how much increase in tissue pO_2 was obtained. In contrast, Ausman et al., (6,9), in experiments on the dog limb, showed that use of blood with an oxygen saturation much below normal improved the results of perfusion, there being a clearly defined relationship between oxygen saturation and net absorption of nitrogen mustard. They contended that the relative hypoxia produced a vasodilatation which enlarged the vascular bed available to nitrogen mustard. They recommended use of a 70% O_2, 30% CO_2 gas mixture to maintain maximum vasodilatation. The relevance of this work on normal dog limbs to tumor tissue is not clear. It has previously been pointed out that blood flow through tumor tissue may be twenty times less than that through the host organ (54), and it may be that vasodilatation of normal tissues would further reduce the relative flow through tumor tissue. However Golomb et al., (49) also doubt the advantage of high pO_2 and report better tumor response in patients with head and neck malignancies using non-oxygenated blood as the perfusate.

In this study, since limb damage was no greater in

group B animals than in group A, it would appear that the use of an oxygenated perfusate is not necessary in isolation perfusion of the rat hind limb, with reference to survival of normal tissues. This may undoubtedly be attributed to the short duration of perfusion. It does, however, appear that increased pO_2 potentiates the action of T.S.P.A. on the Walker tumor. The increase in mean survival time was small but significant. Unfortunately, actual measurements of pO_2 could not be made but a difference of some 500 mmHg in perfusate entering the limb may reasonably be assumed. It would also be advantageous to record tissue pO_2 within the tumor, but such information must await the advent of reliable instrumentation.

The significance of this finding is not great, since it refers only to one transplantable animal tumor and one alkylating agent. It does however lend further support to the views expressed by other authors, that high oxygen tension should potentiate the biological action of alkylating agents. Whether or not the increase in pO_2 achieved by a bubble oxygenator, over that of blood at normal pO_2, has any significant effect remains to be elucidated.

Summary of Part 4.

It was shown that alteration in perfusate temperature did not produce a corresponding alteration in tumor temperature. This finding was attributed to a relatively poor blood flow

through the tumor vascular bed. Consequently there was no significant difference in the response of the Walker tumor to T.S.P.A. at perfusate temperatures of 42°, 37° and 22°C. Diathermy was found to be an unsuitable method of heating deep-seated tumors.

A high perfusate pO_2 was found to increase survival time significantly over that obtained using a perfusate with low pO_2. Use of a low pO_2 did not increase ischemic damage in perfused limbs, probably because the duration of perfusion was short.

CHAPTER IV

A COMPARATIVE STUDY OF SYSTEMIC, INFUSION AND ISOLATION PERFUSION CHEMOTHERAPY USING THE WALKER TUMOR

Klopp (74) and Sullivan (81,128) showed that the intra-arterial administration of nitrogen mustard produced regressive effects on a variety of human neoplasms that were not observed after the intravenous use of this drug. More recently Klopp (76) has compared his results obtained with fractionated intra-arterial nitrogen mustard in carcinoma of the head and neck, with the results reported by Woodhall (140) using regional perfusion, concluding that perfusion for carcinoma of the head and neck offered no real advantage over fractionated intra-arterial therapy. While perhaps true for head and neck tumors this conclusion cannot be generally applied, because the high leakage factor in head and neck perfusions (65% in 30 minutes (66)) prevents the use of drug concentrations much above those customarily employed systemically. As Creech (34) has emphasized, only in the treatment of lesions of the extremities has it been possible to achieve a degree of circulatory isolation permitting a significant increase in the concentration of chemotherapeutic agents without increasing their toxic side-effects.

Creech (34), after four years experience with regional perfusion, has stated that there is no evidence at the present time that the combination of regional chemotherapy and local excision of primary melanoma is more effective than excision alone, although continuing to uphold his earlier claims (113) that regional perfusion in secondary melanoma of the extremities is superior to systemic chemotherapy. Stehlin (123) has obtained similar results in malignant melanoma of the extremity and believes that perfusion may be of value as a therapeutic measure for patients with certain other types of malignant neoplasms. Stehlin is presently collecting a series of cases in which perfusion has been the sole therapy for primary melanoma without clinical evidence of metastases, in an effort to determine the clinical and histologic effects upon such primary lesions.

While both the infusion and isolation perfusion methods of chemotherapy have on occasion caused remarkable instances of tumor regression which would probably not have occurred with conventional systemic chemotherapy (28), the techniques, and particularly isolation perfusion, remain very much experimental procedures and there is a need for extensive, controlled, comparative studies to establish the superiority or otherwise of infusion and perfusion over conventional modes of therapy (57,59). One reason for the present lack of controlled studies has been the wide variation in the techniques employed. Thus different investigators

have perfused similar lesions with different drugs at different concentrations, with or without oxygenation of the perfusate, at varying temperatures and for shorter or longer periods of time. Apart from such purely technical differences however, any attempt to compare systemic, infusion and perfusion chemotherapy from the available literature is rendered hazardous on several counts. In the first place there has been little or no attempt at randomization of cases, presumably because of the moral obligation on the physician to provide the treatment most likely to succeed. Secondly, chemotherapy has been most often tried when all else has failed; thus the lesion may well have been excised and re-excised, irradiated and re-irradiated, so that little uniformity of case material remains. Thirdly and in similar fashion, if one agent failed to produce a response a second was tried in rapid succession, or the two were given in combination or together with irradiation. Finally an accurate evaluation of many cases has proved impossible because the anatomic site of the lesion prevented objective assessment of response, or because the early death of the patient resulted in an inadequate trial.

It seemed therefore that an experimental comparison of systemic, infusion and perfusion chemotherapy might help to resolve the problem. Using small animals it would be possible to achieve uniformity of host sex, size, age and strain, and to assess the effectiveness of the techniques

in terms of the response of a single tumor with a well-established and consistent growth pattern. A study was therefore designed to compare the effects on the Walker tumor of T.S.P.A. administered in the following ways:

1. Systemically, by intravenous injection.
2. Locally, by slow intra-arterial infusion.
3. By isolation perfusion of the tumor-bearing region.
4. By a combined technique of isolation perfusion followed by intra-arterial infusion.

Materials and Methods.

Animals. Male Sprague-Dawley rats weighing 250 to 350 grams were used. The Walker carcinoma was transplanted in the manner described in Chapter II. In all experiments treatment was started on the 4th. day after inoculation of tumor.

Chemotherapeutic Agent. The alkylating agent T.S.P.A. was chosen for this study because our experience had been greatest with this agent. Furthermore the alkylating agents are commonly used for systemic, intra-arterial and perfusion chemotherapy in clinical practice. In the first three experimental groups a total concentration of 4 mgm/kg body weight was used; in the fourth group where perfusion was followed by infusion a total dose of 7 mgm/kg was administered.

Systemic Administration. 54 rats were treated in this group. T.S.P.A. was given intravenously in divided doses on four successive days, each animal thus receiving 1 mgm/kg/day.

The technique for intravenous injection was as follows:-

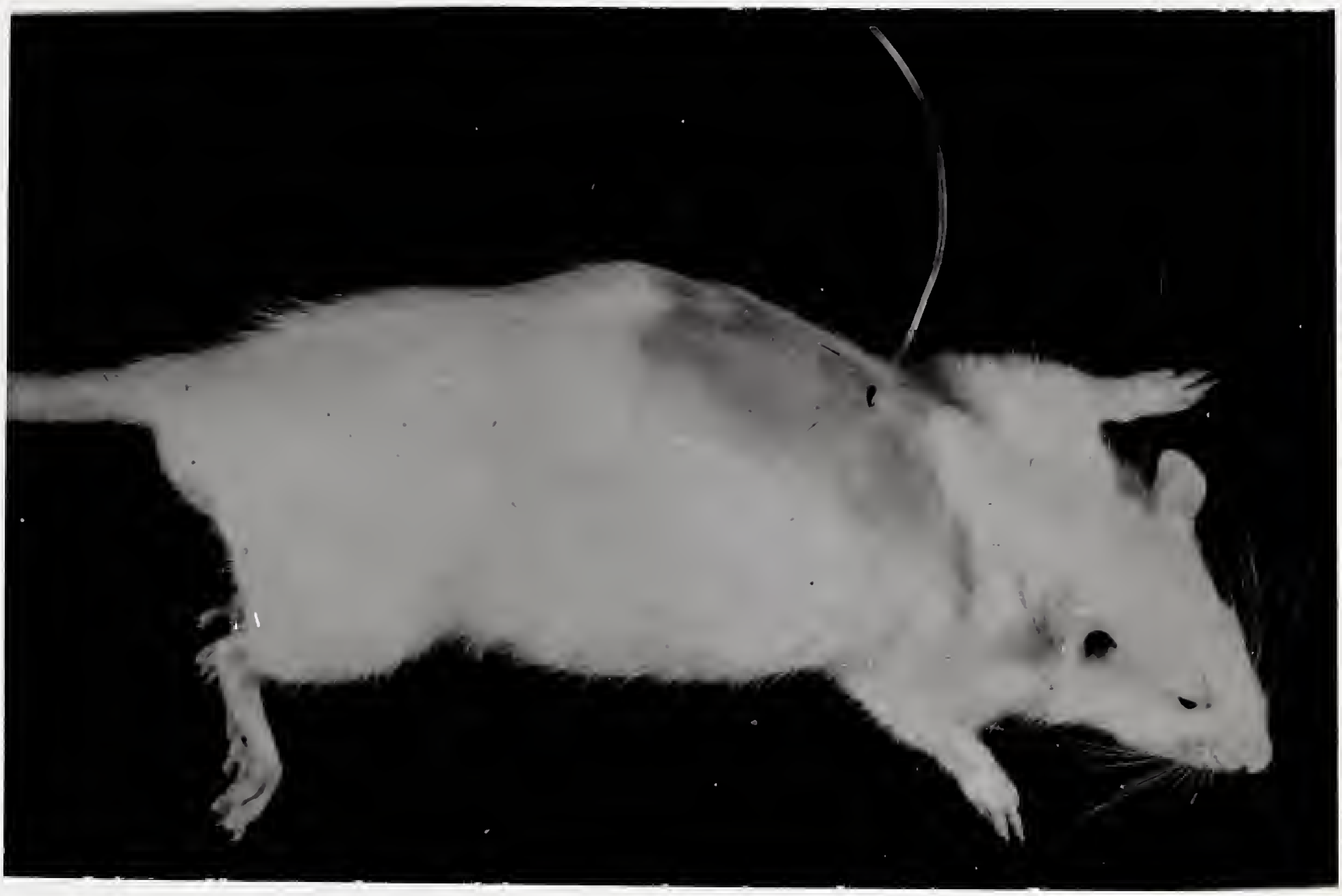
Ether inhalation anesthesia was used. Once anesthetized the animals were placed in a supine position and the non-tumor bearing hind limb shaved over its medial aspect and prepared with 2 1/2% tincture of iodine. A 1 cm incision was made over the saphenous vein on the medial aspect of the lower leg. Using a tuberculin syringe and 27 gauge needle the vein was entered at the lowest point of the incision and the drug injected slowly. After withdrawal of the needle light pressure was maintained over the vein for approximately one minute to prevent bleeding; the skin was then closed with a single metal clip. It was usually possible to give all four injections into the same vein by moving up the vein each day. Occasionally the vein thrombosed and it was necessary to locate and inject some other superficial vein, or to resort to use of the femoral vein.

Intra-arterial Infusion. 58 rats were studied in this group. T.S.P.A. was again administered in divided doses (1 mgm/kg/day) on four successive days, but in this group use was made of a technique for slow intra-arterial infusion:-

The rats were anesthetized with intraperitoneal pentobarbital sodium, 35 mgm/kg body weight. The tumor bearing leg was prepared and the femoral vessels exposed in the manner described for perfusion (Chapter II). The femoral artery was dissected free and ligated proximally. A cannula was prepared from an 8 inch length of P.E. 50 polyethylene tubing. This was filled with a dilute heparin solution

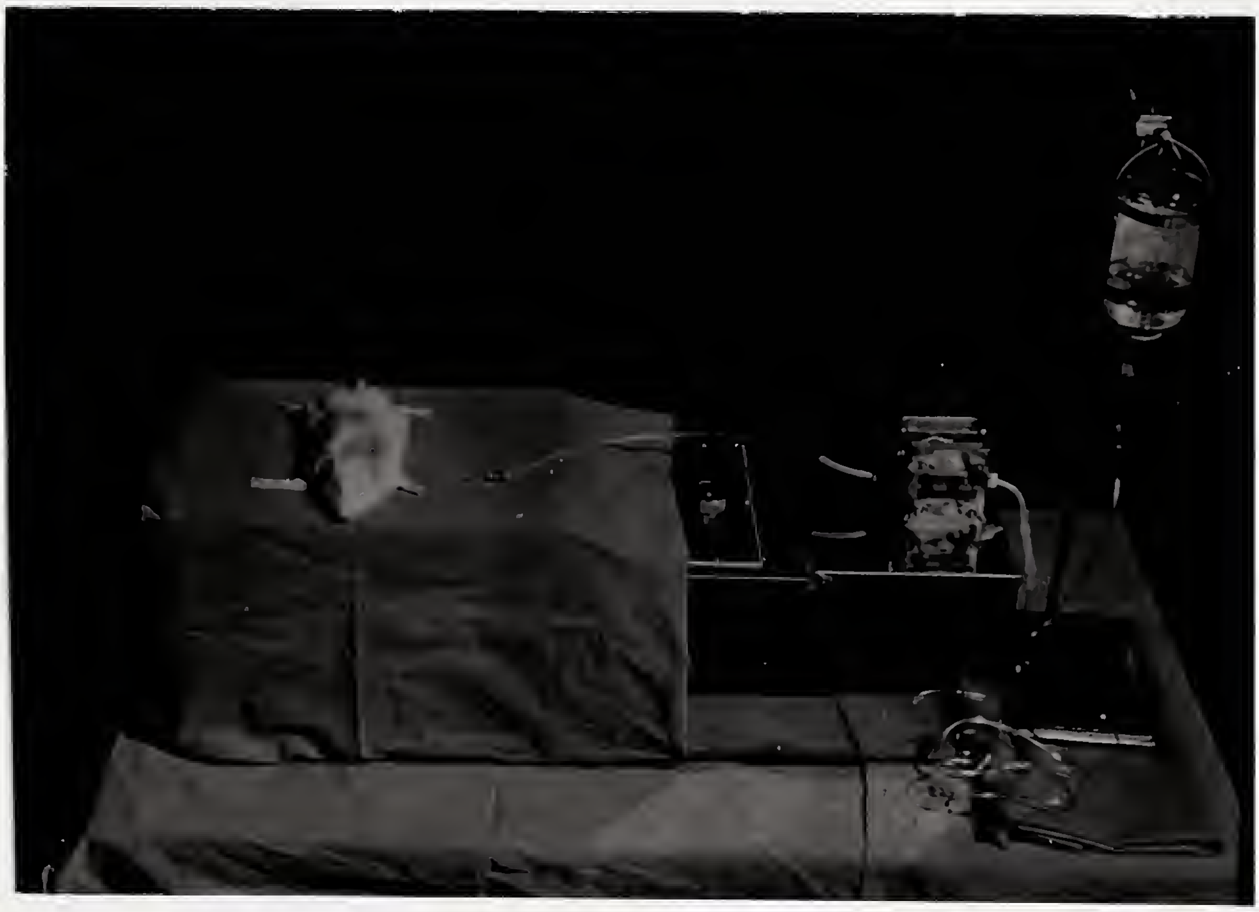
(1 mgm heparin in 1 cc sterile isotonic saline) and one end inserted distally into the femoral artery. A 4-0 silk ligature was tied around the cannulated portion of the artery, and an additional ligature placed around the cannula and artery above the point of cannulation to provide further support A subcutaneous tunnel was made from the cephalad end of the groin incision around the abdomen and thorax to the dorsum of the rat's neck. The cannula was then threaded through the tunnel, leaving sufficient slack in the groin to allow for free movement of the hind limb. A loose tie was placed through the inguinal ligament and around the cannula to maintai the curvature of the cannula in the groin and prevent kinking. The femoral vein was ligated to delay venous emptying from the limb, and the groin incision closed in layers. The animal was then placed in the prone position without any restricting tapes (Fig. 15), and the cannula connected to the pump. A Sigmamotor finger-pump set to deliver 3 cc/hour was used. The T.S.P.A. was diluted in isotonic saline to provide a concentration of 0.1 mgm/cc and placed in a sterile flask from which it was pumped into the cannula (Fig. 16). In this way a 300 gram rat requiring 1 mgm/kg/day would receive 0.3 mgm T.S.P.A. or 3 cc of solution, necessitating infusion for 1 hour. Smaller or larger amounts of T.S.P.A. for rats of different weight were provided by shortening or lengthening the period of infusion. At the conclusion of infusion the cannula was filled with dilute heparin and plugged so that no heparin could leak back. The excess cannula was then

FIGURE 15.



Photograph of rat prior to infusion. The P.E. 50 cannula can be seen emerging from the neck incision.

FIGURE 16.



Rat and equipment during intra-arterial infusion. Flask containing T.S.P.A. to right of photograph. From this flask fluid was pumped at 3 cc/hour into the cannula seen emerging from the dorsal surface of the rat's neck.

coiled and buried subcutaneously, and the neck incision closed with a metal clip.

On succeeding days the rat was again anesthetized, the neck incision reopened and the cannula recovered. The patency of the cannula was checked by gently flushing it through with a small amount of dilute heparin. Very occasionally the cannula was found to be blocked and it was necessary to insert a new cannula. The animal was then connected to the pump and infused as before. It was found better not to tape the animals down; this prevented the animals from struggling should they awaken before completion of the infusion. When the series of four infusions was completed, the cannula was cut short so that the neck end would retract beneath the skin but no attempt was made to remove the cannula.

Perfusion. 53 rats were treated by isolation perfusion of the tumor bearing limb. The standard technique employing an oxygenated perfusate maintained at 37°C was used (see Chapters II and III). Perfusion was continued for 15 minutes, T.S.P.A. 4 mgm/kg being administered in three divided doses at 0, 5 and 10 minutes.

Perfusion Followed by Intra-arterial Infusion. 57 rats were studied in this group. These rats were perfused with T.S.P.A. 4 mgm/kg on the 4th. day after tumor inoculation. The perfusion technique was modified only in that an 8 inch PE 50 cannula, as used for intra-arterial infusion, was substituted for the usual arterial cannula. At the

conclusion of perfusion the venous cannula was removed and the vein ligated; the arterial cannula was tunneled subcutaneously and brought out on the dorsal surface of the rat neck. Thereafter the rats were managed in the manner described for the infusion technique; they were infused on the succeeding three days so that these rats received a total dose of 7 mgm T.S.P.A./kg body weight.

Controls. A total of 105 untreated control rats were observed.

At the conclusion of treatment, or in the case of controls after tumor inoculation, all the animals were kept in individual cages and fed a standard laboratory diet with water ad libitum. No further treatment of any kind was given, the animals being left to die or otherwise from progression of the tumor. Regular examinations were made, particular attention being paid to tumor size and condition and the function of the involved limb. Autopsies were performed on all animals within 24 hours of death and the presence or absence of nodal and pulmonary metastases observed. Metastases were graded in the manner described in Chapter III.

Results.

Animal Survival Time. The survival times resulting from the different techniques of administering T.S.P.A. are shown in

Table VIII. All survival times were calculated from the day of inoculation of tumor. Deaths listed as due to complications were deaths occurring within 24 hours of treatment and attributable to the anesthetic or occasionally to hemorrhage from the groin wound, the latter probably being due to over-heparinization. A few rats were re-anesthetized for photographic purposes; these were removed from the study and are included with the deaths due to complications. A number of rats in each group were "long-term survivors," that is, they survived over 100 days from the day of tumor inoculation. These animals were clinically free of disease, and were re-inoculated with Walker tumor in the contralateral hind limb. In all cases the second inoculation "took" in the usual way.

The results of a statistical analysis of the mean survival times are shown in Table IX. Each technique of administering T.S.P.A. is compared with every other technique. All treatment methods significantly increased survival time when compared with untreated controls, although only a comparison of systemically treated animals and untreated controls is given in Table IX. Both the perfusion and infusion techniques significantly prolonged survival times over those obtained by systemic administration. Perfusion was more effective than infusion.

While the combination of perfusion and infusion was more effective than infusion alone, it was not significantly better than perfusion alone despite the greater

TABLE VIII

Treatment	Number of Rats	T.S.P.A. mgm/kg	Deaths due to complications	Long-term survivors	Number of rats evaluated	Survival (days)	
						Mean	Range
Systemic	54	4	4	3	47	31.8	23-46
Infusion	58	4	3	2	53	36.1	25-60
Perfusion	53	4	0	2	51	38.9	28-53
Perfusion + infusion	57	7	7	4	46	40.7	33-61
No treatment	105	-	-	5	100	21.5	17-32

A comparison of survival times in rats with the Walker tumor treated with T.S.P.A. administered by different techniques. Deaths due to complications were deaths occurring within 24 hours of treatment and attributable to the anesthetic or to hemorrhage from the wound. Long-term survivors were animals surviving over 100 days; these animals were clinically free of disease and were not included in calculations of survival time.

total dose of T.S.P.A. used in the combined technique.

Limb Damage. Positive limb damage was considered to have occurred if:

(a) there was failure of full recovery of normal limb function insofar as could be judged by gross examination;

(b) there was any evidence of muscle wasting;

(c) there was any evidence of ischemic necrosis within the limb. The greatest incidence of limb damage in this study was associated with intra-arterial infusion, either alone or in combination with perfusion (Table X). This finding may reflect the intermittent use of the arterial cannula, small thrombi perhaps being dislodged when the patency of the cannula was checked, since the damage most commonly took the form of necrosis of the tips of one or more digits.

It was of interest to note that in the four long-term survivors following combined perfusion and infusion, there was a marked degree of muscle wasting in the treated limb. This probably resulted from the larger dose of T.S.P.A. either affecting the muscles directly, or secondarily as a consequence of neurotoxicity which is not an uncommon complication following perfusion with alkylating agents (107). However none of the other rats in this group exhibited such a degree of muscle wasting, and it was not clear why the four survivors should be selectively affected in this manner.

Metastases. The primary objective of this study was a comparison of survival times; the metastatic patterns at death were observed only incidentally as part of routine

TABLE IX

TREATMENT	MEAN SURVIVAL IN DAYS	STANDARD DEVIATION	P VALUE
Systemic	31.8	4.707	<0.01
No treatment	21.5	3.740	
Systemic	31.8	4.707	<0.01
Infusion	36.1	5.828	
Systemic	31.8	4.707	<0.01
Perfusion	38.9	5.306	
Systemic	31.8	4.707	<0.01
Perf.+ Inf.	40.7	5.981	
Infusion	36.1	5.828	<0.05
Perfusion	38.9	5.306	
Infusion	36.1	5.828	<0.01
Perf.+ Inf.	40.7	5.981	
Perfusion	38.9	5.306	>0.1
Perf.+ Inf.	40.7	5.981	

Analysis of survival times in rats receiving T.S.P.A. administered by different techniques. P values obtained by two-tailed test of confidence limits for the difference of means in small samples (72). $P < 0.05$ (95% confidence limits) significant.

procedure. For this reason no microscopic studies were performed and the presence or absence of metastases was decided solely on gross examination. Accordingly, no statistical analysis has been made of the data obtained. The results of a microscopic study of metastases are presented in Chapter V. The manner of grading pulmonary and nodal metastases was explained in Chapter III.

The incidence of pulmonary and aortic nodal metastases in the different groups is shown in Table X. Systemic and perfusion chemotherapy reduced the incidence of involved nodes below that found in untreated animals, while infusion, alone or combined with perfusion, resulted in a greater incidence of node involvement. No such distinction was apparent when pulmonary metastases were compared, there being only minor differences between the various groups and the highest incidence occurred in the untreated controls. It can also be seen from Table X that the average grade of pulmonary metastases similarly varied very little between groups. The same did not hold true for the abdominal nodes; infusion, besides increasing the incidence of node involvement, also increased the average size of these nodes at autopsy. In contra-distinction perfusion resulted in fewer nodes of a smaller average size than those found in untreated controls.

Discussion.

This study was performed in an attempt to assess the relative merits of systemic, infusion and perfusion

TABLE X

Treatment	Number of Rats	Incidence Limb Damage %	Abdominal Nodes Incidence %	Abdominal Nodes Grade*	Lungs Incidence %	Lungs Grade*
Systemic	47	0	70.2	2.0	83.0	2.5
Infusion	53	5.7	94.3	3.0	90.6	2.3
Perfusion	51	2.0	66.7	1.9	88.2	2.4
Perf. & Inf.	46	6.5	93.5	3.3	84.8	2.7
No treatment	100	0	82.0	2.4	92.0	2.5

* For explanation of grading of metastases, see Chapter III.

Incidence of limb damage, and metastatic pattern (abdominal nodes and lungs) found at autopsy, in rats receiving T.S.P.A. administered by different techniques.

chemotherapy in a controlled trial. While the methods of systemic and perfusion therapy were analogous to those used in clinical practice, some criticism may be levelled at the technique used for intra-arterial infusion. While resembling Klopp's method (74) for fractionated intra-arterial therapy with nitrogen mustard, it differed in that the drug was given slowly over a one hour period rather than by brief intra-arterial injections. It obviously bore no relation whatever to Sullivan's prolonged infusions with an antimetabolite (129). The rationale for its use was that it provided a means of repeated administration of the drug into a major artery supplying the tumor-bearing region, and since the drug was given over a total period of approximately four hours compared with the fifteen minutes of perfusion, many more cells would in theory be exposed to the anticancer agent during the susceptible mitotic period.

The main conclusion drawn from this study was that regional administration of an alkylating agent, whether by intermittent intra-arterial infusion or by isolation perfusion, was more effective in prolonging survival time than systemic administration. This was undoubtedly due to a greater effect of the agent on the primary lesion. The animals used in this study, with the Walker tumor implanted in the hind limb, die from progressive deterioration of the primary tumor, the terminal event customarily being hemorrhage from an ulcerated, necrotic tumor mass. Very occasionally pulmonary metastases may give rise to a terminal hemothorax. It was certainly the more profound effect of T.S.P.A. on the primary that

prolonged survival when the agent was administered intra-arterially.

It may be suggested that bone marrow depression was responsible for the earlier demise of the systemically treated animals; while no marrow studies were done to permit a positive denial of this criticism, it is thought unlikely that marrow depression was an important factor since the systemically treated animals out-lived untreated controls. In addition all the animals were weighed routinely since weight is a sensitive index of a rat's general health, and the systemically treated animals gained weight in a virtually identical manner to the untreated controls.

Isolation perfusion was a better method of regional chemotherapy than was intra-arterial infusion. The improvement in survival time achieved by perfusion may have been the result of an enhanced activity of the agent consequent upon the use of a warm, oxygenated perfusate. As was mentioned earlier (Chapter III), the increased oxygen tension occasioned by the use of a bubble oxygenator appears to potentiate the effect of radiomimetic agents such as T.S.P.A. It was interesting to find that the addition of infusion to perfusion did not significantly improve survival time, despite a greater total dose of T.S.P.A. and a longer period of exposure. This might suggest that all the susceptible tumor cells were effectively destroyed by the smaller but concentrated dose used in perfusion, while the resistant cells from which subsequent regrowth occurred remained unaffected even by a

larger total dose administered over a longer period of time. As described in Chapter V, there are always small islands of tumor cells apparently untouched by chemotherapy; whether this is a consequence of a genetically determined resistance or merely the result of a fortuitous anatomic location is not clear.

The different metastatic growth patterns after chemotherapy by infusion and perfusion methods are difficult to explain. Why the technique of intermittent intra-arterial infusion should have resulted in an increased incidence and size of aortic nodal metastases, while perfusion did not, is enigmatic. Since the infused animals did not live as long as the perfused animals, the difference could not be a function of duration of growth. For the same reason a greater rate of growth must be assumed, suggesting perhaps some reduction in host resistance to metastatic growth. This may have been the result of repeated and prolonged anesthesia since the infusion technique required that the animals were anesthetized on four separate occasions for periods of greater than one hour. In this regard it should be mentioned that these animals lost weight during the four days of treatment, although they rapidly regained this weight after cessation of therapy. Operative stress in the form of celiotomy has been shown to reduce the resistance of rats to Walker carcinoma (22), resulting in increased "takes" and a faster rate of growth after subcutaneous inoculation of a suspension of tumor cells. In this study the "amount" of surgery was

equally as great in the perfused rats as in the infused group, suggesting that surgical stress per se was not an important factor. It may be that the stress of anesthesia was more significant than the surgery itself. The increased incidence of nodal metastases also requires explanation. It could not be assumed that larger numbers of viable cells escaped from the infused tumors since there was no obvious reason why they should, so once again a reduction in host resistance must be invoked; in this instance the reduced resistance would permit a greater proportion of disseminated cells to become established as metastases. The fact that a similar increase in size and number of pulmonary metastases did not occur in infused rats suggests that the susceptibility of the local "soil" - as postulated in the "soil hypothesis" (104) - should be considered. Reduction in host resistance due to systemic effects of the drug could not have been responsible, since such effects would have been greater in the systemically treated animals which in fact had fewer and smaller metastases.

The increased incidence of limb damage in the infused rats probably resulted from the formation of small thrombi on the cannulae which were dislodged when the patency of the cannula was checked prior to the succeeding infusion. Tying off the major vessels to the limb was not responsible since this has been done many times in normal rats without ill-effect, and is in fact part of the routine procedure after perfusion.

The few long-term survivors that occurred in each group were of interest. These rats were judged to have had successful "takes" after tumor inoculation on the basis of a palpable mass of increasing diameter. Following treatment, or in the case of the controls on the sixth or seventh day after inoculation, the mass began to regress until only a small firm nodule was palpable. No regrowth of these primary tumors occurred. Because it was thought that these might represent spontaneous regressions due to a greater host resistance in these animals, the opposite hind limb was subsequently inoculated with tumor. In all cases the second inoculation "took" and tumor growth progressed in the usual way, resulting in the deaths of these animals within the customary period taken for untreated controls to die. It therefore appeared that these were not spontaneous regressions due to a heightened host resistance, but were merely unsuccessful "takes", the palpable mass perhaps being of a simple inflammatory nature. The incidence of unsuccessful "takes" was remarkably uniform from group to group.

Summary.

Isolation perfusion was the most effective method of treating the Walker 256 carcinoma, implanted in the right hind limb of rats, with T.S.P.A. Survival time was longer than after systemic therapy or intermittent intra-arterial infusion, and the incidence of limb damage was less than that

resulting from infusion. The incidence and extent of metastases were similar in the systemically treated and the perfused animals. Infusion resulted in more numerous and larger aortic nodal metastases.

CHAPTER V

THE HISTOPATHOLOGY AND METASTATIC SPREAD OF WALKER 256 CARCINOMA TREATED BY ISOLATION PERFUSION WITH T.S.P.A.

Although regional perfusion chemotherapy of "solid" tumors has been in use in a number of centers since 1957, there have been very few reports concerning the histopathologic changes produced by perfusion with alkylating agents. There have been no reported studies, at the time of writing, on the influence of perfusion of the primary lesion on metastatic spread.

Stehlin et al., (120) reported on the histologic changes in malignant melanoma perfused with phenylalanine mustard. The microscopic appearance of these tumors after perfusion revealed necrosis in which the necrotic shadow cells retained the pattern of the pre-existing melanoma. Continued degeneration was exhibited by the presence of amorphous granular debris and, in some cases, by liquefaction necrosis. These changes were limited to the tumor itself and were sharply delineated from the surrounding non-neoplastic tissue, demonstrating a distinct selectivity of the drug for the tumor. Necrosis was apparent in almost all the metastatic tumors examined histologically. Several lesions were completely necrotic, but in others viable tumor cells were

present at the periphery. In a further paper, Stehlin (119) mentioned that some of the tumors exhibited microscopic evidence of spontaneous necrosis pre-operatively and as a consequence proper evaluation of necrosis after perfusion was difficult.

Creech et al., (31) again treating metastatic melanoma of the extremity with phenylalanine mustard, described extensive infiltration of the lesion with small mononuclear cells which appeared to break up the neoplastic cells into islands and, eventually, the entire metastasis was replaced by fibrous connective tissue. They believe that phenylalanine mustard, in addition to destroying neoplastic cells directly, also initiates certain changes which make these cells more susceptible to resistance factors of the host. This assumption is based primarily on the observation that regressive changes within the neoplasm continued long after the drug had become inactive, and after the period of acute cellular necrosis resulting from the chemotherapeutic agent had passed.

Koller (79) described the action of both alkylating agents and radiation as being injury to chromosomes with excessive fragmentation and breakdown of the mitotic mechanism. He suggested that these changes may be the result of metabolic disturbances. He further illustrated that inhibition of growth in experimental tumors was not brought about by radiomimetic injuries alone; other events such as suppression of

mitosis, cell pyknosis in the resting stage, and stromal reaction also played a role. In earlier experiments Koller and Casarini (78) had compared the effects of X-rays and nitrogen mustard on 6 and 7 day old Walker tumors grafted subcutaneously into white albino rats. After nitrogen mustard, the proportion of injured cells increased with time, so that 144 hours after treatment about 90% of the dividing cells exhibited chromosomal injury in the form of highly atypical mitoses, with disorganization of the chromosome mechanism and giant cell formation. Following radiation, cellular injury decreased after 12 hours and was almost absent 72 hours after treatment. From the data obtained Koller concluded that nitrogen mustard acted on the resting stage chromosome filament either before or during the process of synthesis, while X-rays acted directly on definite linkages of the already synthesized polypeptide structure of the prophase chromosome thread.

Sternberg et al., (125) described changes in the mesenteric lymph nodes of normal Wistar rats given nitrogen mustard. These changes consisted of a decrease in size of the node, a loss or blurring of the follicular pattern, and a disappearance of germinal centers. There was a concomitant diminution in the number of lymphocytes at the end of three days.

The study to be described was undertaken in order to determine the effect of T.S.P.A. on the cells of the

Walker carcinoma under the conditions of the standard perfusion. In addition, histologic proof was needed to confirm macroscopic evidence of metastasis.

It is convenient at this point to describe the normal histology of the Walker tumor. The tumor originally developed spontaneously in the region of the mammary gland of a pregnant albino rat in 1928 in the laboratory of George Walker at the Johns Hopkins University School of Medicine (127). The first two transplant generations of the tumor had only limited success. Thereafter the transplant tumors grew successfully in nearly 100% rats receiving intramuscular or subcutaneous injections of tissue fragments.

Histologically the tumor was originally diagnosed as an adenocarcinoma with both simple and complex acini and loose cellular connective tissue containing slender spindle-shaped cells with elongated hyperchromatic nuclei. A third cell type had a somewhat larger granular nucleus with a relatively prominent nucleolus. The current tumor is composed of all three cell types, the epithelioid type of cell predominating (Figs. 17 & 18). At first the young transplant is chiefly carcinomatous, but as it grows older it develops a sarcomatous pattern. The spindle-shaped cells resemble neoplastic fibroblasts and appear to favor the periphery of the tumor.

It was mentioned in Chapter II that the tumor has been variously termed a carcinosarcoma, sarcoma or simply just a tumor. While it is true that both carcinomatous

and sarcomatous elements are present, it is referred to here as a carcinoma because it was originally described as such by Earle (36) and the recent studies by Fisher and Fisher (43) are in agreement with this designation. They demonstrated effacement of cell borders, a lack of demonstrable protoplasmic fibrillary extensions and an argyrophilic reticulum pattern, all features consonant with an epithelial nature. They further pointed out that even the sarcomatous components were tinctorially, morphologically and ultrastructurally similar to the more clearly carcinomatous areas. The presence of transitional forms between the spindle and round or polygonal tumor cells also indicated that the former represented morphologic variants of the more common prototype.

Method of Study.

72 male Sprague-Dawley rats weighing 200 to 300 grams were inoculated with a suspension of Walker carcinoma cells in the manner described in Chapter II. 36 rats (AP 1 to 18 and BP 1 to 18) were perfused with T.S.P.A., 4 mgm/kg, on the 4th. day after tumor inoculation. The conditions of the standard perfusion obtained. The remaining 36 rats (AC 1 to 18 and BC 1 to 18) remained untreated as controls.

At predetermined intervals 4 perfused and 4 control rats were killed by guillotine. These intervals,

in days after inoculation of tumor, are shown in Tables XI and XII. Complete autopsies were performed on all rats. Sections were taken of the primary tumor or its site of inoculation, of the abdominal para-aortic lymph nodes and of the lungs from all rats. A gross assessment of the presence or absence of metastases was made and recorded, metastases being graded 0 to 4+ as described in Chapter III.

Between the time of tumor inoculation and sacrifice, all rats were kept in individual cages and fed a standard laboratory diet with water ad libitum. No further treatment was given to any rat. Many of the control rats died a "natural" death from progression of the tumor before the intended time of sacrifice. Three perfused rats died similarly, and one died post-operatively for reasons unknown. While alive all rats were examined at two-day intervals, their weight, general health and tumor size being recorded.

All specimens were fixed in 10% formalin, sectioned at 5 microns in duplicate and then stained with hematoxylin and eosin.

Results.

Tables XI and XII summarize the observations made.

Macroscopically, the most obvious difference between the perfused and control animals was in the size of the tumor. In the controls the maximum diameter of 5.5 cm was reached by the 17th. day. In the perfused group there was no increase

in diameter from the 7th. to the 17th. day, and diameters in the 4 cm range were not reached until 31 days after tumor inoculation. Similarly, and with few exceptions, metastases occurred in the controls by the 17th. day but were delayed in the perfused group until the 31st. day. Thus macroscopic evidence suggested that growth and spread of the tumor was delayed for a period of 14 days by perfusion with T.S.P.A. This was confirmed by the histologic findings.

Histologically, by the 7th. day the control tumors exhibited focal areas of degeneration. These degenerative changes consisted of nuclear pyknosis and increased intensity of staining of the cytoplasm, with decrease in overall size of the cell. The 7th. day perfused tumors (3 days post-perfusion), however, showed actual coagulation necrosis rather than degeneration (Fig. 19), with preserved cells located at the periphery and in immediate perivascular areas. In both the perfused and control tumors relatively frequent regular mitotic figures were present in the degenerated and necrotic areas as well as in the more intact regions.

As the tumor progressed degeneration became more evident in the control tumors, and there was evidence of actual necrosis by the 11th. day (Figs. 20 & 21). In the perfused tumors, necrosis was no more extensive by the 11th. day than on the 7th. day, but numerous huge giant cells were seen. Many of these contained highly atypical mitotic

figures (Fig. 22). These giant cells were from 4 to 8 times the size of the usual tumor epithelioid cells, and occurred particularly in the more preserved areas of the tumor (Fig. 23). Similar giant cells were not seen in any of the control tumors (Fig. 24). Giant cells were still present in the perfused tumors at the 17th. day but were less frequently the site of mitotic activity (Fig. 25). Their numbers then gradually diminished, so that by the 31st. day none were present. At this time the perfused tumors contained relatively few mitotic figures and resembled control tumors of about the 17th. day (Fig. 26).

The location and nature of the metastatic tumors was quite similar in both the perfused and control animals, apart from the different time intervals. There was good correlation between the gross and microscopic evidence of metastasis. In the lymph nodes, the first histologic evidence of tumor consisted of small focal collections of predominantly epithelioid cells (Fig. 27). With increasing time there was complete replacement of all node architecture. In the lungs, the tumors were predominantly sub-pleural in nature (Fig. 28), and in only a few instances was tumor found within the parenchyma itself. Again, the secondary tumor in the lung consisted primarily of the epithelioid type of tumor cell.

An interesting observation was that large active germinal follicles developed in the lymph nodes of control

rats 7 days after inoculation with tumor (Fig. 29). These follicles were not seen in the lymph nodes of normal (non-tumor) rats, and did not occur until the 17th. day in rats having tumors perfused with T.S.P.A. (Fig. 30).

6 tumors regressed spontaneously in the perfused group and 4 in the control group. In all cases these rats had definite evidence of tumor take by the 4th. day, but no evidence of tumor from the 31st. day on. Microscopically 3 of the tumor sites (2 perfused, 1 control) showed residual areas of micronecrosis (Fig. 31).

Discussion.

These studies clearly demonstrate that T.S.P.A. administered by regional perfusion to 4 day old Walker tumors in rats, did not destroy the tumor but resulted in sufficient alterations to inhibit its growth for a period of about 14 days. This inhibition of growth was achieved in several ways. In the first place, large areas of tumor were completely necrosed; this necrosis must be attributed to the chemotherapeutic agent since necrosis was less extensive and occurred later in control tumors. Secondly, the development of huge tumor giant cells with highly atypical mitotic figures 6 to 7 days after perfusion indicated that the chemotherapeutic agent had some deleterious effect upon the chromosomal structure of the nuclei, presumably an effect on DNA (3). It was only after the disappearance of these highly atypical cells that further increase in the size of

the tumor and metastases occurred. It appeared therefore, to take about 14 days for the tumor to completely overcome the toxic effects of T.S.P.A. and to revert to a normal histologic and biologic pattern.

The good correlation between gross and microscopic evidence of metastasis was important, because in all the studies previously described only gross estimates of metastasis were made. As a result of this work such estimates would appear reliable.

The occurrence of spontaneous regressions of the primary tumor was mentioned earlier in this thesis and discussed in Chapter IV. It was pointed out that it was not possible to distinguish clearly between unsuccessful "takes" and true spontaneous regression of tumors that had "taken". Histologic examination of the site of tumor inoculation did not clarify the situation, merely indicating in a few cases that tumor cells were present but necrotic.

In only one rat was damage to normal tissues evident after perfusion. This was a small area of muscle infarction in relation to a minute focus of tumor necrosis in one of the cases of tumor regression.

The variation in follicular structure of the lymph nodes may indicate that follicular hyperplasia represents some type of host response to the non-perfused tumor, and that alterations in the tumor produced by the chemotherapeutic agent modify the host response so that follicular hyperplasia

is delayed until resumption of growth of the primary tumor. Alternatively the chemotherapeutic agent may have some direct effect on the lymph node itself as was described by Sternberg et al., (125) in the case of nitrogen mustard therapy. The first of these two alternatives must be favored in the light of the evidence presented in Chapter III that leakage from the perfused circuit was negligible.

Summary.

The effect of isolation perfusion of the Walker 256 carcinoma with T.S.P.A. was studied by macroscopic and microscopic examination in 36 treated rats and 36 controls. The results indicated that tumor growth and spread was inhibited for approximately 14 days by perfusion. This resulted from destruction of a large number of tumor cells and interference with the chromosomal structure of many others. Some cells remained intact. Some evidence of a possible host defence mechanism was seen in the form of a follicular hyperplasia in the lymph nodes 7 days after inoculation of tumor in untreated rats.

TABLE 11

CONTROLS

Day after Inoculation	Rat No.	Tumor Size (cm)	Abdominal Nodes		Lungs		Comment
			Gross	Micro.	Gross	Micro.	
4	AC 1	1.3	0	negative	0	negative	PCN ++
	AC 2	1.4	0	negative	0	negative	PCN ++
	AC 3	1.4	0	negative	0	negative	PCN ++
	AC 4	1.4	0	negative	0	negative	PCN ++
5	AC 5	1.7	0	negative	0	negative	PCN ++
	AC 6	1.9	0	negative	0	negative	PCN ++
	AC 7	1.5	0	negative	0	negative	PCN ++
	AC 8	1.8	0	negative	0	negative	PCN ++
7	AC 9	2.5	0	negative	0	negative	PCN FH DGN
	AC 10	1.7	0	negative	0	negative	PCN FH DGN
	AC 11	2.3	0	negative	0	negative	PCN FH DGN
	AC 12	2.1	0	negative	0	negative	PCN FH DGN
11	AC 13	0.9	0	negative	0	negative	NEC FH
	AC 14	2.6	0	negative	1+	negative	NEC FH
	AC 15	4.2	0	negative	0	negative	NEC
	AC 16	2.6	0	negative	0	negative	NEC
14	AC 17	3.0	0	positive	0	negative	FH
	AC 18	2.6	0	negative	1+	negative	FH +
17	BC 1	4.0	1+	positive	3+	positive	FH +
	BC 2	5.5	0	positive	1+	positive	FH +
	BC 3	5.2	3+	positive	2+	positive	FH +
	BC 4	4.5	4+	positive	2+	positive	FH +
18	BC 10	4.9	4+	positive	3+	positive	ND
21	BC 12	4.8	3+	positive	1+	positive	ND
23	BC 6	5.2	2+	positive	2+	positive	ND
	BC 11	4.6	3+	positive	4+	positive	ND
	BC 18	5.6	4+	positive	4+	positive	ND
24	BC 17	4.0	2+	positive	1+	positive	ND
25	BC 7	5.6	4+	positive	2+	positive	ND
	BC 9	4.6	3+	positive	4+	positive	ND
26	BC 16	5.2	3+	positive	0	positive	ND
31	BC 5	R	0	negative	0	negative	MN
	BC 8	R	0	negative	0	negative	FH
	BC 13	R	0	negative	0	negative	FH
38	BC 14	R	0	negative	0	negative	FH
	BC 15	R	0	negative	0	negative	FH

Summary of observations made on rats with untreated Walker carcinoma killed at intervals after tumor inoculation.
Symbols: PCN, plasma cells in nodes. FH, follicular hyperplasia. DGN, tumor degeneration. NEC, tumor necrosis. ND, natural death. R, spontaneous regression. MN, micronecrosis of tumor cells.

TABLE 12

PERFUSED

Day after Inoculation	Rat No.	Tumor Size (cm)	Abdominal Nodes Gross	Abdominal Nodes Micro.	Lungs Gross	Lungs Micro.	Comment
5	AP 1	1.4	0	negative	0	negative	PCN
	AP 2	1.4	0	negative	0	negative	PCN
	AP 3	1.6	0	negative	0	negative	PCN
	AP 4	1.7	0	negative	0	negative	PCN
	BP 14	1.7	0	negative	0	negative	Post-op death
7	AP 5	1.4	0	negative	0	negative	PCN + NEC
	AP 6	1.3	0	negative	0	negative	PCN + NEC
	AP 7	1.4	0	negative	0	negative	PCN + NEC
	AP 8	1.2	0	negative	0	negative	PCN + NEC
11	AP 9	1.1	0	negative	0	negative	GCM
	AP 10	1.3	0	negative	0	negative	GCM FH
	AP 11	1.0	0	negative	0	negative	GCM
	AP 12	1.0	0	negative	0	negative	GCM
14	AP 13	1.2	0	negative	0	negative	GCM
	AP 14	1.0	0	negative	0	negative	GCM
	AP 15	1.2	0	negative	0	negative	GCM FH
	AP 16	1.5	0	negative	0	negative	GCM
17	BP 1	1.8	0	negative	0	negative	GC FH
	BP 2	1.1	0	negative	0	negative	GC
	BP 3	1.0	0	negative	0	negative	GC FH
	BP 4	1.8	0	positive	0	negative	GC FH
24	BP 5	2.1	0	negative	0	negative	MIT FH
	BP 6	2.5	0	negative	0	negative	MIT FH GC
	BP 7	R	0	negative	0	negative	MN FH
	BP 8	R	0	negative	0	negative	FH
31	BP 9	4.6	4+	positive	2+	positive	MIT
	BP 10	3.7	1+	positive	4+	positive	MIT
	BP 11	4.1	2+	positive	2+	positive	MIT
	BP 12	2.7	1+	positive	0	negative	MIT
34	BP 13	4.6	4+	positive	2+	positive	ND
35	AP 17	1.9	4+	positive	0	negative	ND
	BP 16	2.6	4+	positive	1+	positive	ND
42	BP 15	R	0	negative	0	negative	FH
	BP 17	R	0	negative	0	negative	FH
	BP 18	R	0	negative	0	negative	FH
43	AP 18	R	0	negative	0	negative	FH MN

Summary of observations made on rats with Walker carcinoma treated by regional perfusion with T.S.P.A.
Symbols: PCN, plasma cells in nodes. NEC, tumor necrosis. GCM, giant cells containing mitoses. GC, giant cells without mitoses. FH, follicular hyperplasia. MIT, mitoses evident in tumor. MN, micronecrosis of tumor cells. ND, natural death. R, spontaneous regression.

Figure 17. Section of Walker carcinoma 7 days after inoculation into posterior muscle mass of rat hind limb, showing infiltration of the characteristic cells around skeletal muscle fibers at the periphery of the tumor. H & E. X 125.

Figure 18. Section of Walker carcinoma 7 days after inoculation showing the characteristic epithelioid and spindle-shaped cells. H & E. X 500.

FIGURE 17.

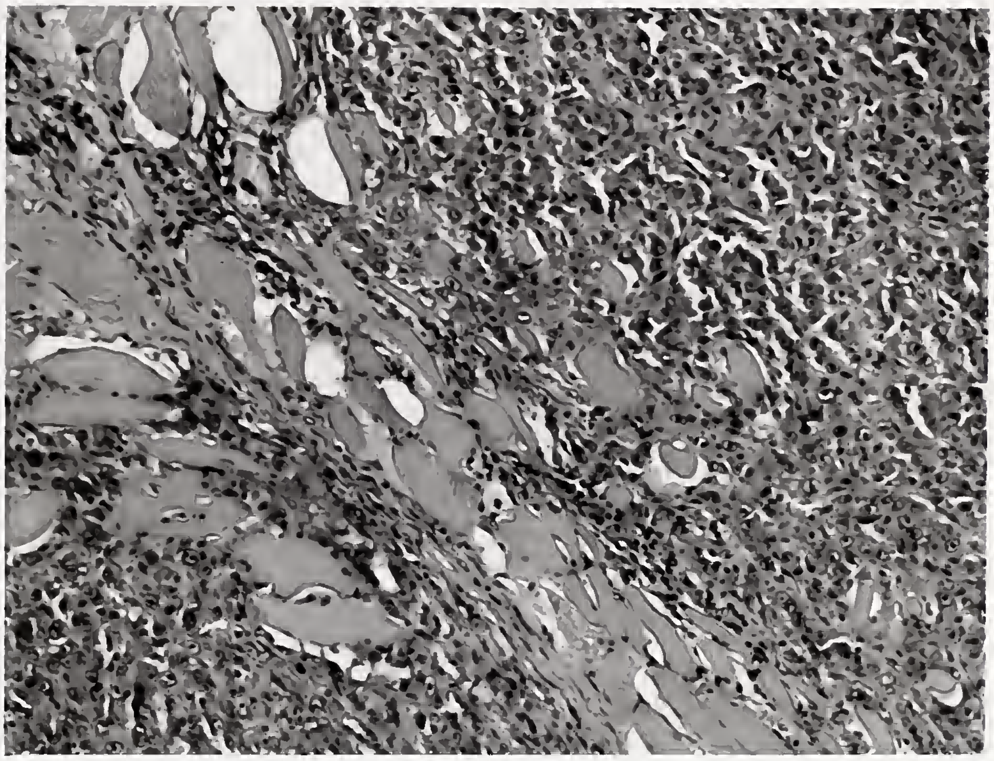


FIGURE 18.

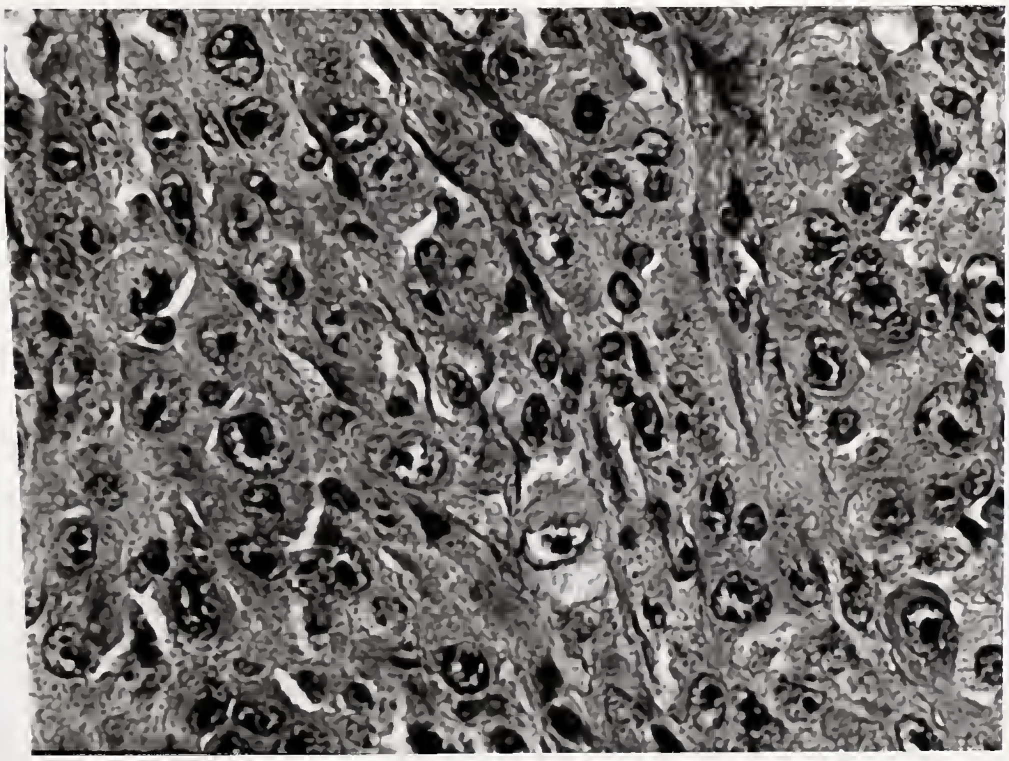


Figure 19. Section of Walker carcinoma from rat 7 days after inoculation and 3 days after perfusion, showing areas of vacuolar degeneration and necrosis of the malignant cells. H & E. X 125.

Figure 20. Section of Walker carcinoma from rat 11 days after inoculation showing large areas of necrosis. H & E. X 125.

FIGURE 19.

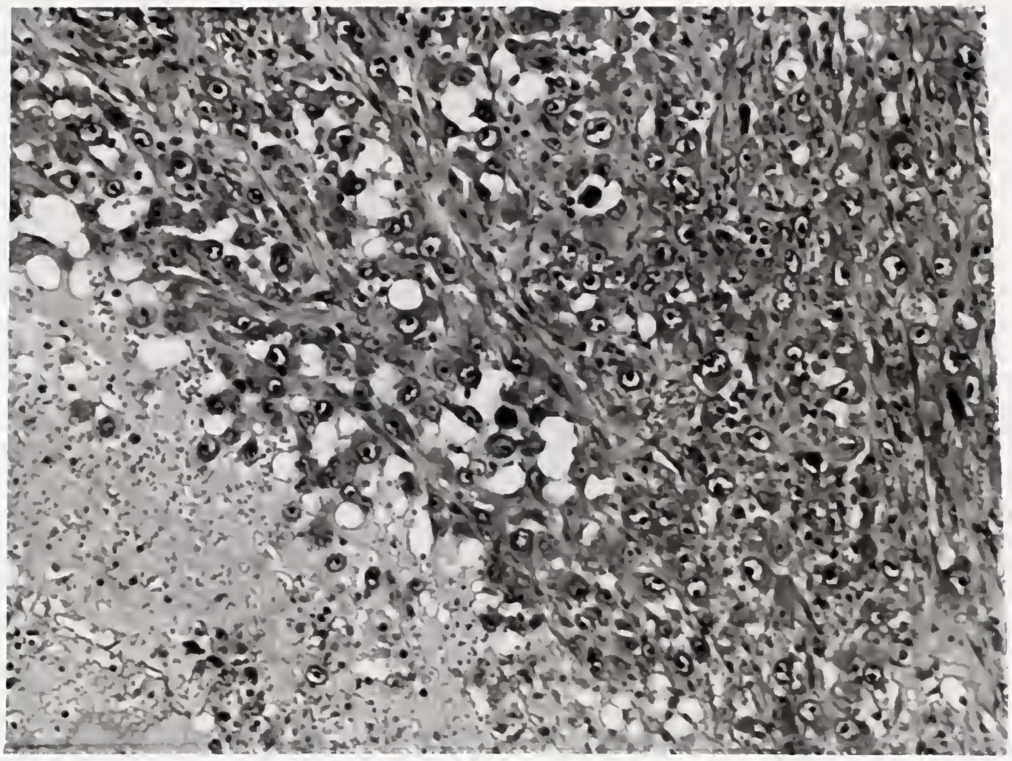


FIGURE 20.

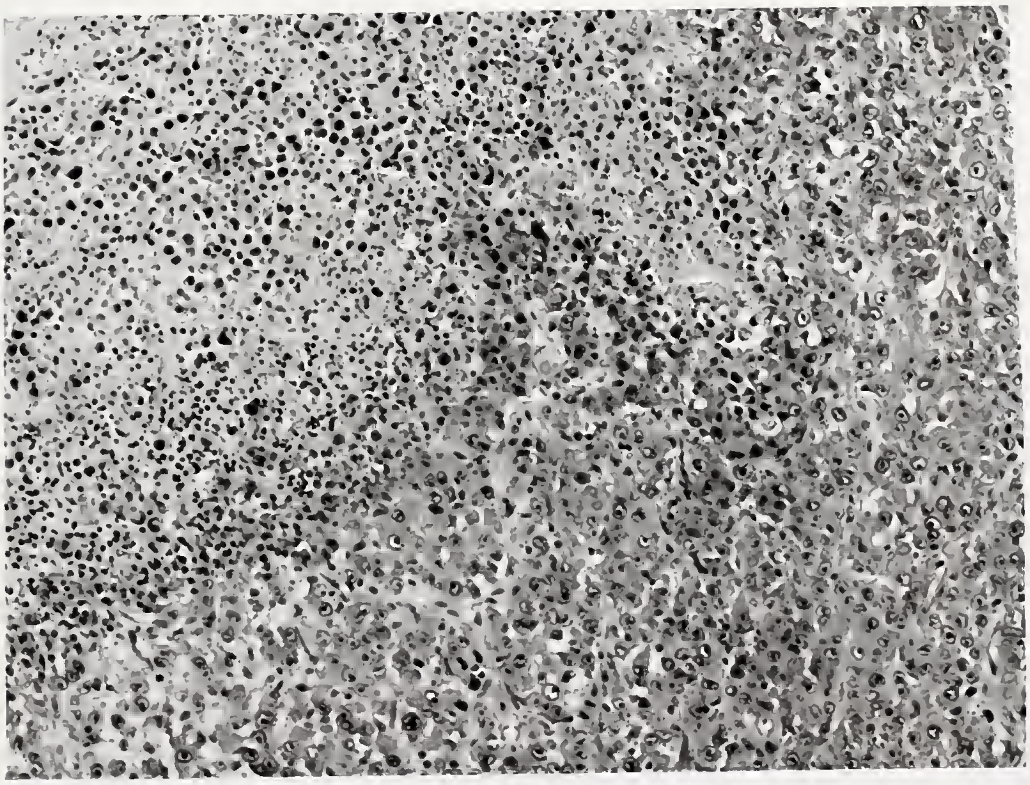


Figure 21. Section of Walker carcinoma 11 days after inoculation showing transition zone between the typical tumor cells and necrotic areas. H & E. X 500.

Figure 22. Section of Walker carcinoma 11 days after inoculation and 7 days after perfusion, showing the presence of huge atypical giant tumor cells. There is a highly atypical mitotic figure in the center one. H & E. X 500.

FIGURE 21.

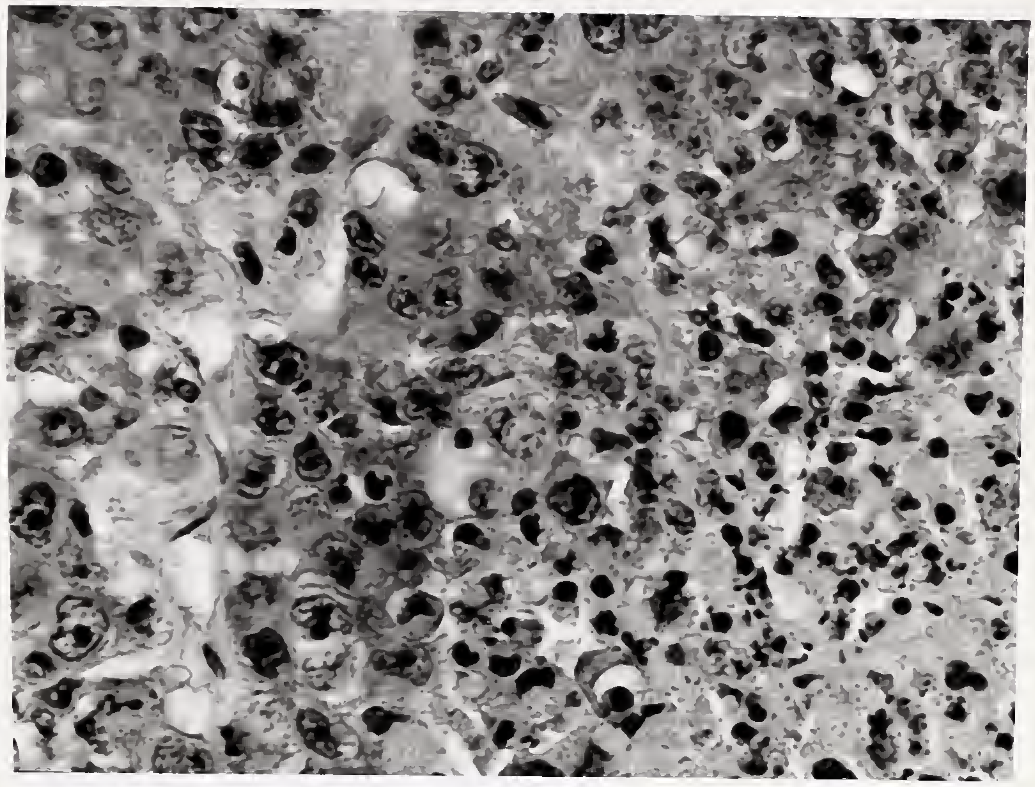


FIGURE 22.

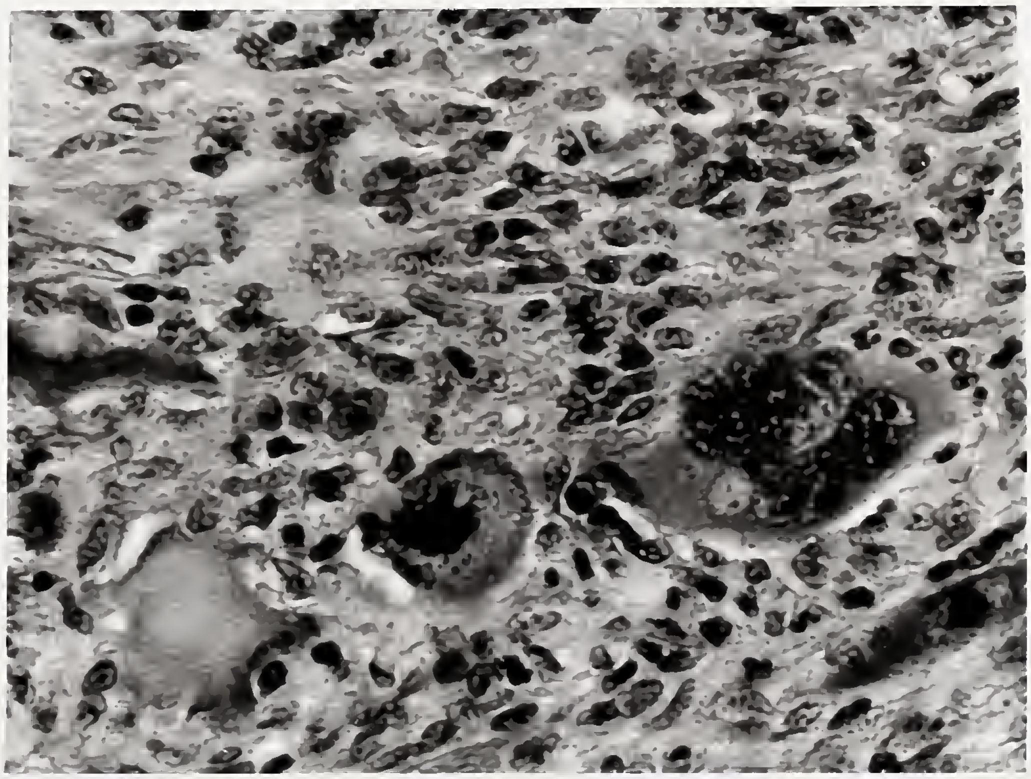


Figure 23. Section of Walker carcinoma 17 days after inoculation and 13 days after perfusion, showing somewhat degenerated tumor cells and one huge irregular tumor giant cell. H & E. X 500.

Figure 24. Section of Walker carcinoma 17 days after inoculation revealing extensive necrosis but absence of tumor giant cells. H & E. X 500.

FIGURE 23.

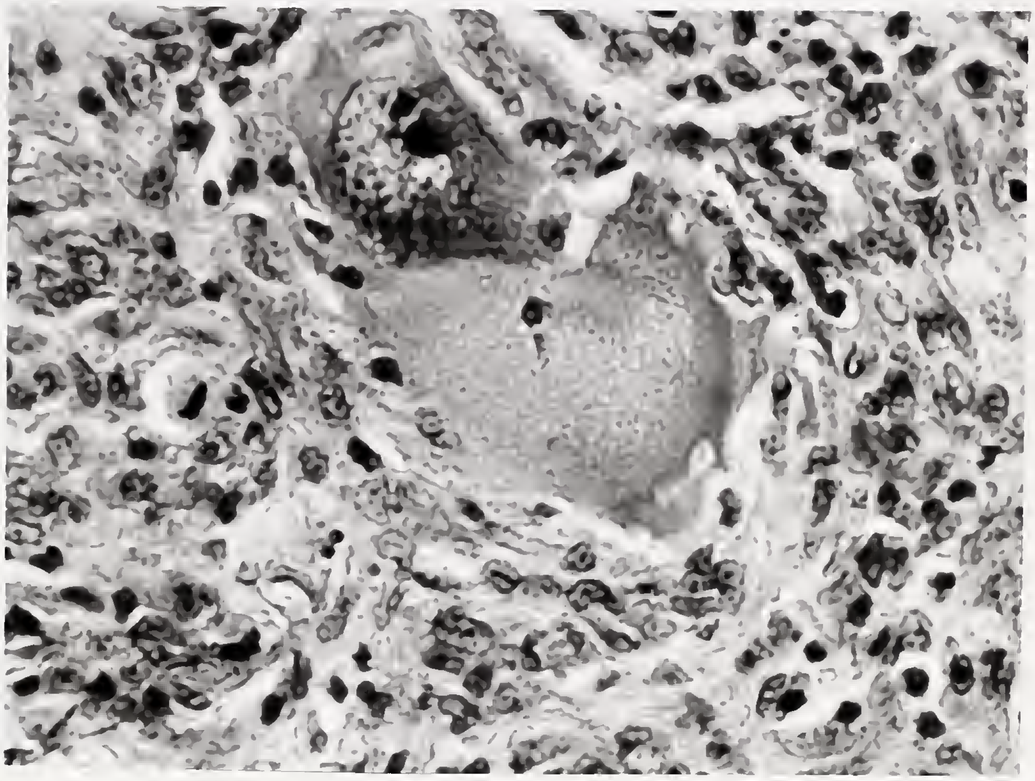


FIGURE 24.

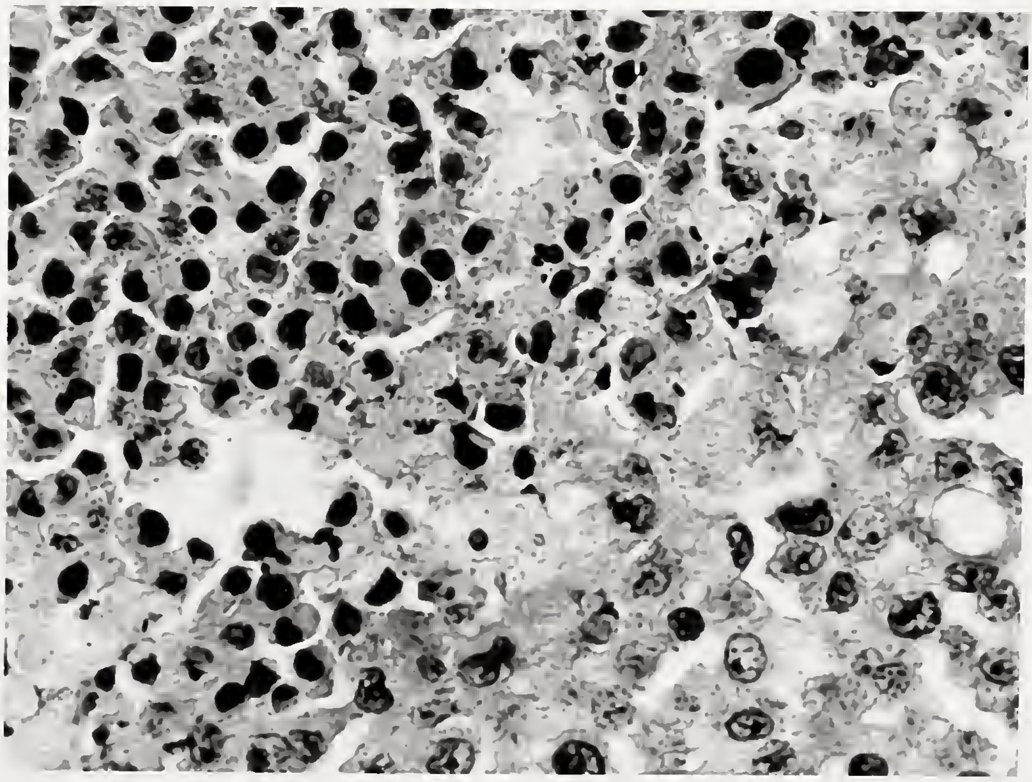


Figure 25. Section of Walker carcinoma 17 days after inoculation and 13 days after perfusion, showing very frequent tumor giant cells but little mitotic activity. H & E. X 125.

Figure 26. Section of Walker carcinoma 31 days after inoculation and 27 days after perfusion, showing the typical Walker carcinoma cells as seen in the non-perfused tumors on the 17th. day. H & E. X 500.

FIGURE 25.

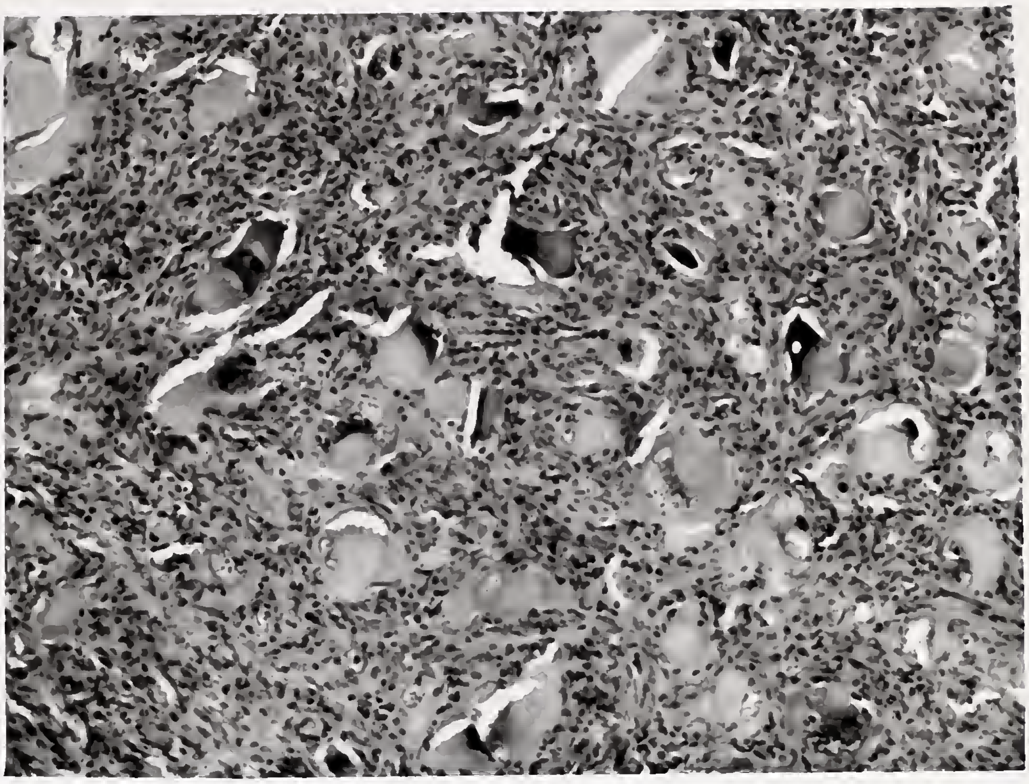


FIGURE 26.

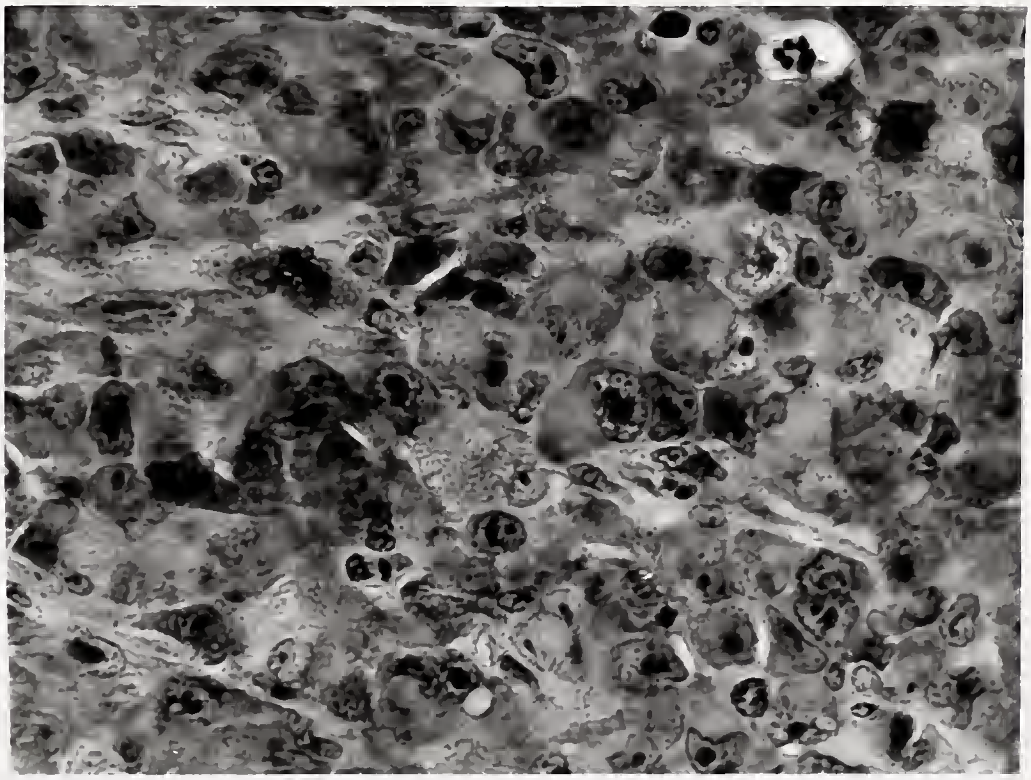


Figure 27. Section of para-aortic lymph node from rat 17 days after inoculation of Walker carcinoma into hind limb muscles, showing lymphocytes in peripheral sinus and a mass of much larger malignant cells in the cortex. H & E. X 125.

Figure 28. Section of lung from rat 17 days after inoculation of Walker carcinoma into hind limb muscles, showing a sub-pleural nodule composed of metastatic malignant cells. The adjacent lung parenchyma is not unusual. H & E. X 50.

FIGURE 27.

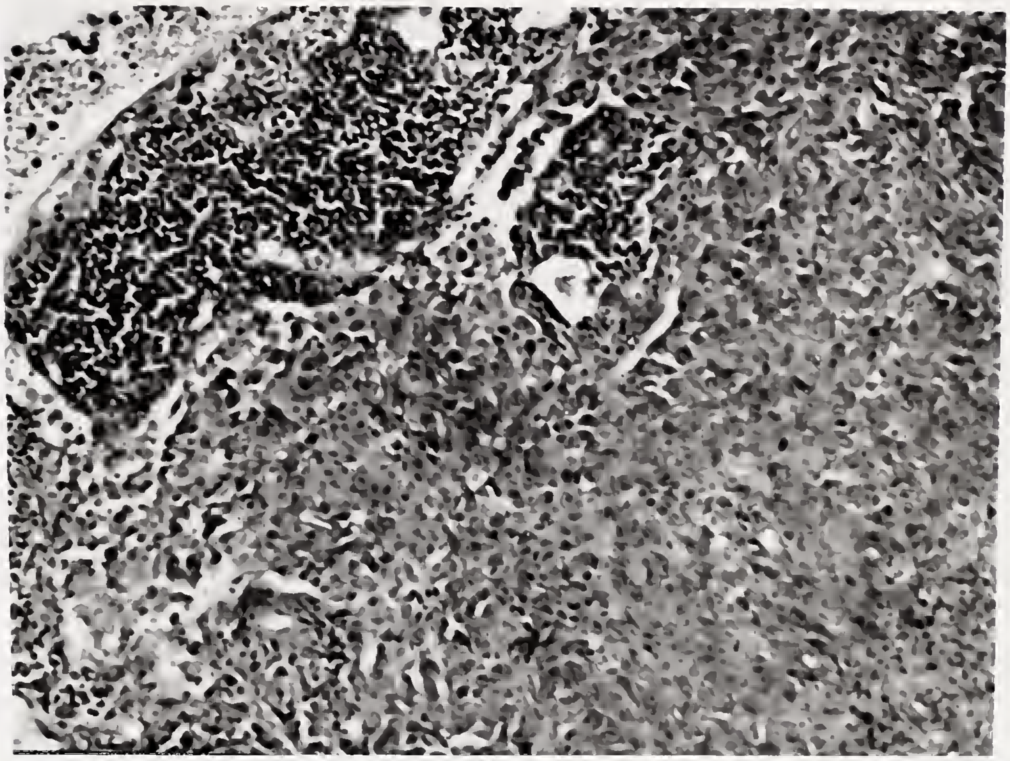


FIGURE 28.

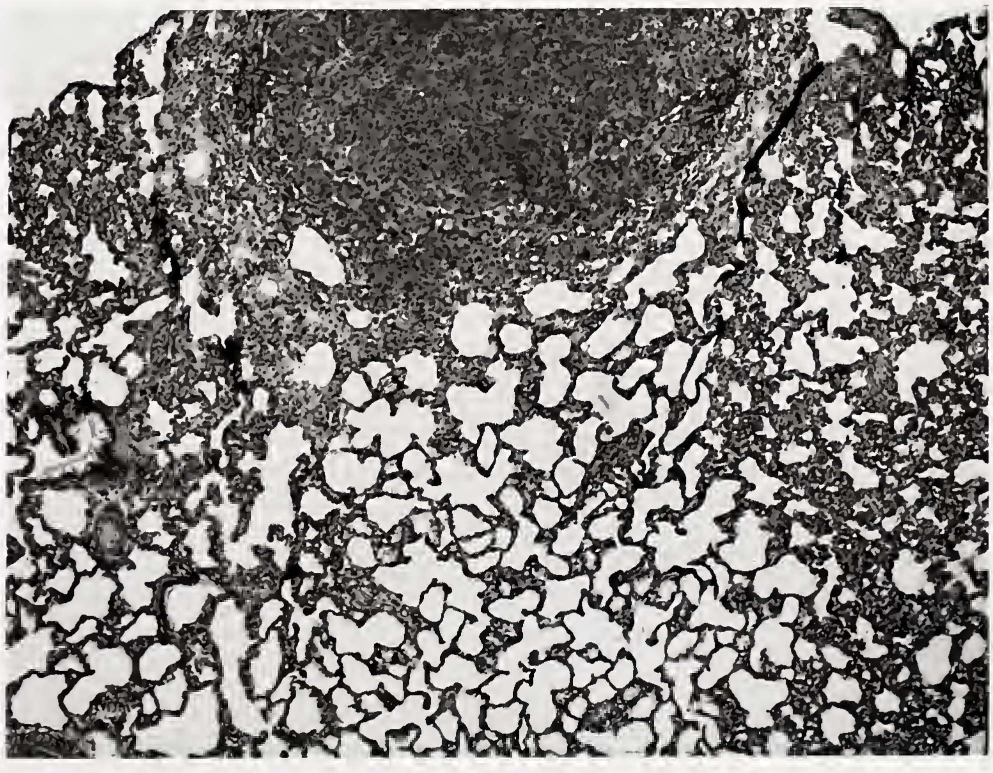


Figure 29. Section of para-aortic lymph node from rat 7 days after inoculation of Walker carcinoma into hind limb muscles, showing the presence of numerous large lymph follicles composed of relatively pale epithelioid type cells. H & E. X 125.

Figure 30. Section of para-aortic lymph node from rat 7 days after inoculation of Walker carcinoma into hind limb muscles and 3 days after perfusion, showing absence of lymph follicles. H & E. X 125.

FIGURE 29.

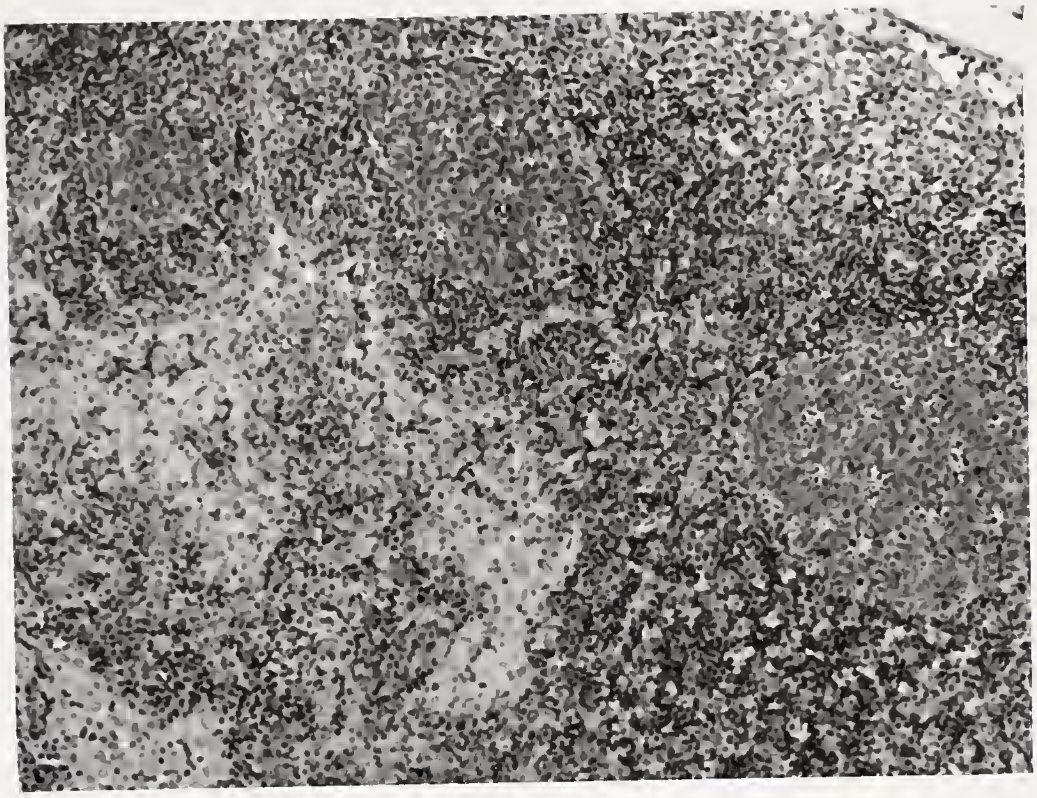


FIGURE 30.

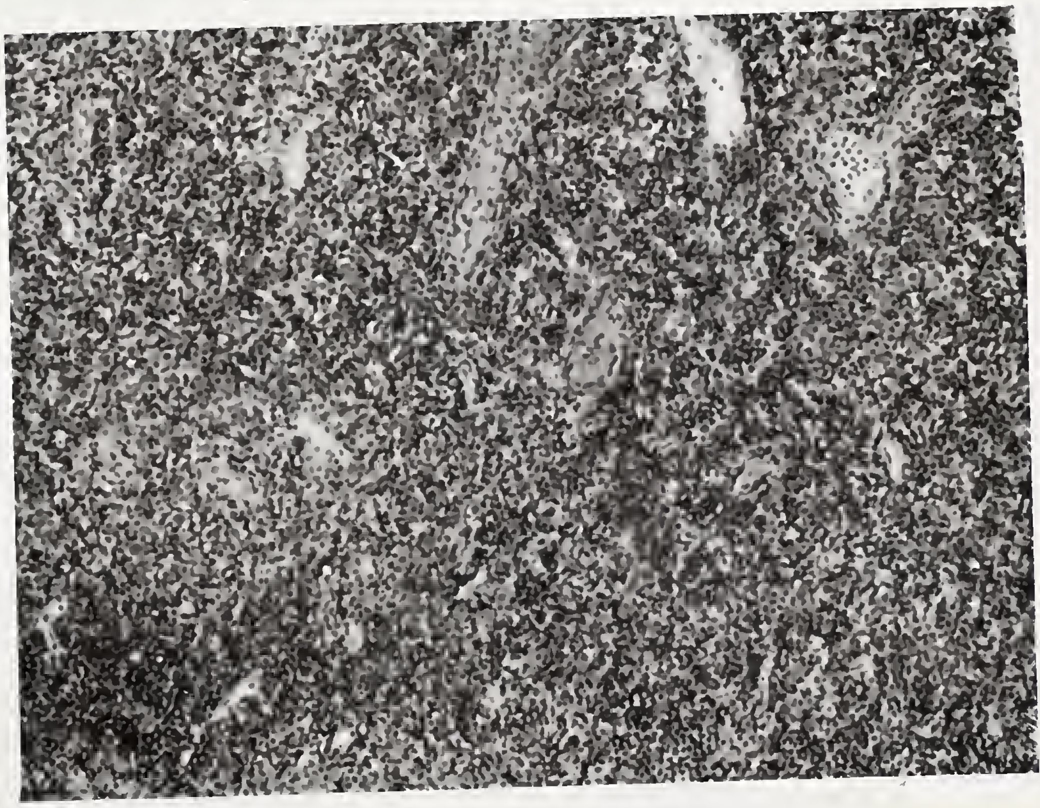
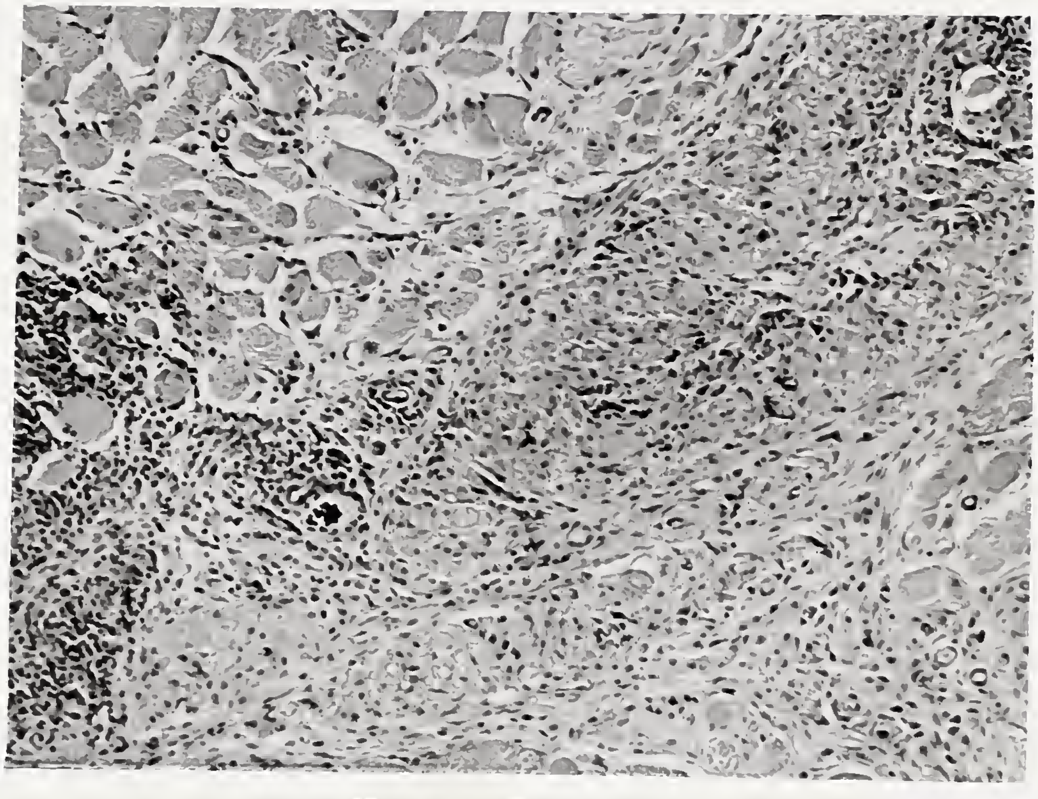


Figure 31. Section of thigh muscle of rat in region of inoculation of Walker carcinoma cells 31 days previously, showing a microscopic focus of chronic inflammatory cell infiltration but intact skeletal muscle fibers.

H & E. X 125.

FIGURE 31.



CHAPTER VI

CLINICAL AND EXPERIMENTAL REGIONAL CHEMOTHERAPY OF CANCER. A SUMMARY.

In the introduction to this thesis, it was stated that the stimulus towards initiating this work was the lack of a suitable experimental preparation for studying the many variables related to perfusion in terms of the response of a well-characterized tumor. The technique for isolation perfusion of the rat hind limb bearing the Walker carcinoma would appear to have satisfied this requirement. It has, however, become apparent since the inception of this work that regional chemotherapy, particularly by isolation perfusion, may ultimately be of limited value in clinical practice, and its use perhaps confined to a small number of responsive neoplasms in anatomic regions that can be well isolated. Thus Herter et al., (61) were forced to conclude after experience with 48 patients treated by perfusion that: "The clinical results.... are disappointing. Of the 48 patients, a handful only have demonstrated tumor regression, and in these, with rare exception, the improvement has been transitory. Complications have been infrequent but serious."

Henschke and Cole (59), after reviewing the results reported in the literature, stated: "The reports show that regression of tumors which do not respond to a systemic administration of chemotherapeutic agent can be accomplished with perfusion techniques. However, we doubt that the results achieved to-day by perfusion surpass the accomplishments of good local radiation therapy, as is claimed by Creech and his associates for melanoma which is usually labelled "radioresistant". We believe that a controlled clinical study is required before a claim of superior results with perfusion in melanoma can be accepted." Similar views have been expressed concerning intra-arterial use of nitrogen mustard by Bonner et al., (19), Boiman et al., (18) and by Grady et al., (50) who concluded that: "... the occasional palliation obtained does not warrant the time consuming and vigorous procedure, symptomatology accompanying therapy and the attendant complications."

The major theoretic advantage of perfusion is the ability to deliver to a tumor-bearing region concentrations of drugs with a narrow therapeutic range that would be lethal if given systemically. With good isolation of the part, the only factor limiting dose is the local tissue tolerance. This advantage is of proven value in perfusion of the extremity with melanoma, the agent of choice (phenylalanine mustard) producing usually, but not always, a good response in this way (34,123), whereas systemically administered phenylalanine mustard rarely results in tumor regression and

its use is fraught with danger (61). Unfortunately such favorable responses are in the minority when they are compared with the total number of all cancers treated by perfusion. An additional and unforeseen advantage of regional chemotherapy has been the symptomatic relief afforded many of these patients, in particular the relief of intractable pain (66, 115). Pain relief is often immediate and complete, suggesting a direct action of the agent on sensory nerves (motor nerves are rarely affected) rather than a secondary action due to regression in tumor size (61). Again, and unhappily, this response is usually transitory.

The major limitation of perfusion lies with the difficulty in achieving good vascular isolation. Without this, the advantage of the technique is lost, It is unfortunately true that with the exception of extremity perfusions, good isolation is rarely achieved. This necessitates short perfusions and use of drugs such as nitrogen mustard that are rapidly inactivated. Long-acting drugs such as T.S.P.A., which remains active in blood for at least 3 hours (33), would obviously be hazardous if perfusion was continued for any length of time with a high degree of leak. Herter et al., (61) have called attention to another potential danger of perfusion therapy. In several of their patients with melanoma, tumor growth

appeared to have been accelerated by perfusion, perhaps because of some alteration in local host defence factors. This finding contrasts with the opinion of Creech and his associates (34,83) who suggest that confinement of the drug to the isolated part will prevent interference with host defence mechanisms; they further claim that the response of melanomas is potentiated due to an enhanced antigenicity of the tumor and stimulation of a more vigorous antibody response. It is difficult to reconcile these opposing views, but it is noteworthy that recent studies by Kondo and Moore (80) have shown that, in animals, pretreatment with a number of carcinostatic agents greatly enhances the growth of transplanted ascites tumor cells; these observations serve as a warning that under some circumstances chemotherapy may be worse than no treatment at all. A further hazard of perfusion that has been described is the occasional rapid destruction of large tumors resulting in serious sequelae due to release of toxic protein breakdown products. The technical limitations of perfusion have been largely discussed in the preceding chapters.

It is a verisimilitude that chemotherapy is only as good as the agents available. Edelman et al., (37) believe that the future of perfusion depends on the discovery of more selective agents in which the relationship of tumor-inhibitory action, binding to tumor tissue, and systemic toxicity are in

better balance. It is the author's belief that with the advent of selective agents the burdensome technique of isolation perfusion will have no place in chemotherapy, for it is unreasonable to expect a large tumor mass to respond completely to but a few minutes of concentrated drug therapy. For this reason the work of Sullivan et al., (129) has particular appeal. Their technique of prolonged intra-arterial infusion of an antimetabolite while preventing systemic toxicity with intermittent systemic administration of the specific metabolite, offers a method of prolonged exposure of the tumor to anticancer agents, so that the majority, if not all, of the malignant cells are exposed during the susceptible mitotic period. It may also be that combinations of drugs may exert synergistic or at least additive effects and prevent the emergence of resistant cell lines, while the combined use of chemotherapy and irradiation may also be of value (11). These latter methods of approach are presently under study (77,90,97,137).

It has also become apparent that there is little specificity of presently available agents for a given tumor (33). For this reason efforts are being made to develop sensitivity tests in much the same way as these are used in microbial chemotherapy. These techniques involve establishing tissue cultures of biopsy specimens of human tumors, and exposing them to chemotherapeutic agents at concentrations

comparable to those which could be achieved in the human (95). Such techniques remain largely in the experimental stage. Isolation perfusion of the rat hind limb does not lend itself to sensitivity testing, but may be of value as an initial screening test for new chemotherapeutic agents. Failure of such agents to produce a satisfactory tumor response at the high concentrations made possible by isolation perfusion, would deem success unlikely at ordinary systemic dose levels.

Before summarizing the main content of this thesis, it is pertinent to discuss the relevance, if any, that perfusion of Walker tumor in rats might have to the problem of human neoplasia. The objection undoubtedly may be raised that the response of a transplantable animal tumor does not necessarily reflect the response of a spontaneous human tumor. It is true that transplantable animal tumors can serve only poorly as a physiological model for cancer in man, but the need in these experiments was for reproducibility. This study was in no sense a study of basic cellular biochemistry or physiology, but was concerned in a general way with tumor response to chemotherapeutic agents administered in different concentrations for different periods of time, and the effect on such response of alterations in tumor environment. With this in mind and emphasis on the need for reproducibility, it is not unreasonable to extrapolate the

results of animal studies to clinical usage, provided that such results are accepted with the necessary reservations, and regarded as only indicative of the probable findings. If this premise is accepted, then the value of the work done can be assessed and the possible future role of the technique described.

To summarize. A technique has been established whereby the circulation of the hind limb of rats may be isolated from the general body circulation. The isolated limb may be successfully perfused with donor rat blood at pressures below systemic pressure, so that the leak of perfused blood into the systemic circulation is negligible. Controlled alteration of the biochemical and physiologic conditions of perfusion is possible. Addition of chemotherapeutic agent to the perfusate, and inoculation of Walker carcinoma cells into the muscles of the limb, permitted study of the effect of alteration of these controllables on the response to that agent of a well-characterized tumor.

It was established that the effect of perfusion of 4 day old Walker tumors with an alkylating agent (T.S.P.A., 4 mgm/kg rat body weight) was to increase animal survival time from 21 to 39 days. Both grossly and histologically there was suppression of tumor growth for approximately 14 days after perfusion. Subsequent tumor growth progressed in characteristic fashion, and metastasized so that the metastatic pattern at death was essentially similar to that

in untreated animals.

Studies on variation of perfusate temperature were inconclusive, but suggested that the actual rate of blood flow may be relatively small through tumors which are characterized histologically as highly vascular. The use of externally applied heat in the form of diathermy was shown to be of no value when heating deep-seated tumors. A high perfusate oxygen tension was shown to be effective in potentiating the action of T.S.P.A. on the Walker tumor.

In a controlled, comparative study of systemic administration, intermittent intra-arterial infusion and perfusion chemotherapy, the latter was shown to exert the most profound effect on the tumor as measured in terms of animal survival time. Some variation in the metastatic pattern was seen in this study, but may have reflected the conditions of the experiment rather than the basic concept of the modality of treatment involved.

With regard to the future, the technique may be used to further studies on the effect of environmental alteration. Thus the effects of alteration in pH and pCO_2 should be studied, and much investigation is needed on tumor blood flow and methods to improve this. Combinations of chemotherapeutic agents should be explored for a synergistic or additive effect, while combining radiotherapy with perfusion of chemotherapeutic agents or agents which will potentiate radiation (77) may easily be investigated with this technique.

Other tumors in other animals require to be investigated to lend weight to the validity of findings in rats with the Walker carcinoma. Prolonged intra-arterial infusion of antimetabolites should be tried and compared with other techniques of chemotherapy.

Such studies will take time. Similar studies in man will take much longer.

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